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Search Results - Record(s) 1 through 10 of 37 returned.

☐ 1. Document ID: US 20020146717 A1

L3: Entry 1 of 37 File: PGPB Oct 10, 2002

PGPUB-DOCUMENT-NUMBER: 20020146717
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20020146717 A1

TITLE: Compositions, methods and kits for determining the presence of cryptosporidium parvum organisms in a test sample

PUBLICATION-DATE: October 10, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Cunningham, Melissa M.	Gresham	OR	US	
Stull, Paul D.	San Diego	CA	US	
Weisburg, William G.	San Diego	CA	US	

US-CL-CURRENT: 435/6; 536/23.7, 536/24.3

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw Desc | Image

☐ 2. Document ID: US 20020102568 A1

L3: Entry 2 of 37 File: PGPB Aug 1, 2002

PGPUB-DOCUMENT-NUMBER: 20020102568
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20020102568 A1

TITLE: Nucleic acid sensor molecules

PUBLICATION-DATE: August 1, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Usman, Nassim	Lafayette	CO	US	
McSwiggen, James A.	Boulder	CO	US	
Zinnen, Shawn	Denver	CO	US	
Seiwert, Scott	Lyons	CO	US	
Haeberli, Peter	Berthoud	CO	US	
Chowrira, Bharat	Broomfield	CO	US	
Blatt, Lawrence	Boulder	CO	US	
Vaish, Narendra K.	Boulder	CO	US	

US-CL-CURRENT: 435/6; 536/24.3

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 3. Document ID: US 20020076735 A1

L3: Entry 3 of 37 File: PGPB Jun 20, 2002

PGPUB-DOCUMENT-NUMBER: 20020076735
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20020076735 A1

TITLE: Diagnostic and therapeutic methods using molecules differentially expressed in cancer cells

PUBLICATION-DATE: June 20, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Williams, Lewis T.	Tiburon	CA	US	
Escobedo, Jaime	Alamo	CA	US	
Innis, Michael A.	Moraga	CA	US	
Garcia, Pablo Dominguez	San Francisco	CA	US	
Sudduth-Klinger, Julie	Kensington	CA	US	
Reinhard, Christoph	Alameda	CA	US	
Giese, Klaus	Berlin	CA	DE	
Randazzo, Filippo	San Francisco	CA	US	
Kennedy, Giulia C.	San Francisco	CA	US	
Pot, David	San Francisco	CA	US	
Kassam, Altaf	Oakland	CA	US	
Lamson, George	Moraga	CA	US	
Drmanac, Radoje	Palo Alto	CA	US	
Crkvenjakov, Radomir	Sunnyvale	CA	US	
Dickson, Mark	Hollister	CA	US	
Drmanac, Snezana	Palo Alto	CA	US	
Labat, Ivan	San Francisco	CA	US	
Leshkowitz, Dena	Kiryat Hasavionim	CA	IL	
Kita, David	Foster City	CA	US	
Garcia, Veronica	Sunnyvale	CA	US	
Jones, Lee William	Sunnyvale		US	
Stache-Crain, Birgit	Sunnyvale		US	

US-CL-CURRENT: 435/7.23; 435/6

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 4. Document ID: US 20020055116 A1

L3: Entry 4 of 37 File: PGPB May 9, 2002

PGPUB-DOCUMENT-NUMBER: 20020055116
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20020055116 A1

TITLE: Compositions, methods and kits for determining the presence of cryptosporidium organisms in a test sample

PUBLICATION-DATE: May 9, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Cunningham, Melissa M.	Gresham	OR	US	
Stull, Paul D.	San Diego	CA	US	
Weisburg, William G.	San Diego	CA	US	

US-CL-CURRENT: 435/6; 536/24.3

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC | Draw Desc | Image

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☐ 5. Document ID: US 20010039014 A1

L3: Entry 5 of 37 File: PGPB Nov 8, 2001

PGPUB-DOCUMENT-NUMBER: 20010039014
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20010039014 A1

TITLE: Integrated systems and methods for diversity generation and screening

PUBLICATION-DATE: November 8, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Bass, Steven H.	Hillsborough	CA	US	
Davis, S. Christopher	San Francisco	CA	US	
Patten, Phillip A.	Menlo Park	CA	US	
Tobin, Matthew	San Jose	CA	US	
Minshull, Jeremy	Menlo Park	CA	US	
Welch, Mark	Fremont	CA	US	
Gustafsson, Claes	Belmont	CA	US	
Carr, Brian	Fremont	CA	US	
Jenne, Stephane	Burlingame	CA	US	
Raillard, Sun Ai	Mountain View	CA	US	
Crameri, Andreas	Reinach	CA	CH	
Stemmer, Willem P.C.	Los Gatos	CA	US	
Emig, Robin	Redwood City	CA	US	
Longchamp, Pascal	East Palo Alto	CA	US	
Goldman, Stanley	Walnut Creek	CA	US	
Giver, Lorraine J.	Santa Clara	NV	US	
Affholter, Joseph A.	Lake Village Zephyr Cove		US	

US-CL-CURRENT: 435/6; 435/287.2, 702/20

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 6. Document ID: US 20010031470 A1

L3: Entry 6 of 37 File: PGPB Oct 18, 2001

PGPUB-DOCUMENT-NUMBER: 20010031470
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20010031470 A1

TITLE: Detection of nucleic acid hybrids

PUBLICATION-DATE: October 18, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Shultz, John William	Verona	WI	US	
Lewis, Martin K.	Madison	WI	US	
Leippe, Donna	Madison	WI	US	
Mandrekar, Michelle	Oregon	WI	US	
Kephart, Daniel	Cottage Grove	WI	US	
Rhodes, Richard Byron	Madison	WI	US	
Andrews, Christine Ann	Cottage Grove	WI	US	
Hartnett, James Robert	Madison	WI	US	
Gu, Trent	Madison	WI	US	
Olson, Ryan J.	Madison	WI	US	
Wood, Keith V.	Madison	WI	US	
Welch, Roy	Palo Alto	CA	US	

US-CL-CURRENT: 435/6; 435/91.1

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 7. Document ID: US 20010023063 A1

L3: Entry 7 of 37 File: PGPB Sep 20, 2001

PGPUB-DOCUMENT-NUMBER: 20010023063
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20010023063 A1

TITLE: ASSAYS EMPLOYING ELECTROCHEMILUMINESCENT LABELS AND ELECTROCHEMILUMINESCENCE QUENCHERS

PUBLICATION-DATE: September 20, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
RICHTER, MARK M.	SPRINGFIELD	MO	US	
POWELL, MICHAEL J.	DANVILLE	CA	US	
BELISLE, CHRISTOPHER M.	CONCORD	CA	US	

US-CL-CURRENT: 435/6

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
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☐ 8. Document ID: US 6458945 B1

L3: Entry 8 of 37 File: USPT Oct 1, 2002

US-PAT-NO: 6458945
DOCUMENT-IDENTIFIER: US 6458945 B1

TITLE: Method for analyzing polynucleotides

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 9. Document ID: US 6458543 B1

L3: Entry 9 of 37 File: USPT Oct 1, 2002

US-PAT-NO: 6458543
DOCUMENT-IDENTIFIER: US 6458543 B1

TITLE: Nucleic acid ligand diagnostic biochip

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 10. Document ID: US 6458535 B1

L3: Entry 10 of 37 File: USPT Oct 1, 2002

US-PAT-NO: 6458535
DOCUMENT-IDENTIFIER: US 6458535 B1

TITLE: Detection of nucleic acids by multiple sequential invasive cleavages 02

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
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Term	Documents
DETECT$	0
DETECT.DWPI,EPAB,JPAB,USPT,PGPB.	969845
DETECTA.DWPI,EPAB,JPAB,USPT,PGPB.	11
DETECTAB.DWPI,EPAB,JPAB,USPT,PGPB.	1
DETECTABALE.DWPI,EPAB,JPAB,USPT,PGPB.	6
DETECTABCLE.DWPI,EPAB,JPAB,USPT,PGPB.	1
DETECTABE.DWPI,EPAB,JPAB,USPT,PGPB.	7
DETECTABEL.DWPI,EPAB,JPAB,USPT,PGPB.	4
DETECTABIE.DWPI,EPAB,JPAB,USPT,PGPB.	5
DETECTABILI.DWPI,EPAB,JPAB,USPT,PGPB.	4
DETECTABILILTY.DWPI,EPAB,JPAB,USPT,PGPB.	1
(L2 AND (DETECT$ SAME CATALYT$)).USPT,PGPB,JPAB,EPAB,DWPI.	37

There are more results than shown above. Click here to view the entire set.

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Search Results - Record(s) 31 through 37 of 37 returned.

☐ 31. Document ID: US 5985557 A

L3: Entry 31 of 37 File: USPT Nov 16, 1999

US-PAT-NO: 5985557
DOCUMENT-IDENTIFIER: US 5985557 A

TITLE: Invasive cleavage of nucleic acids

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC | Draw Desc | Image

☐ 32. Document ID: US 5985548 A

L3: Entry 32 of 37 File: USPT Nov 16, 1999

US-PAT-NO: 5985548
DOCUMENT-IDENTIFIER: US 5985548 A

TITLE: Amplification of assay reporters by nucleic acid replication

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC | Draw Desc | Image

☐ 33. Document ID: US 5747252 A

L3: Entry 33 of 37 File: USPT May 5, 1998

US-PAT-NO: 5747252
DOCUMENT-IDENTIFIER: US 5747252 A

TITLE: Nucleic acid probes and amplification oligonucleotides for Neisseria species

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC | Draw Desc | Image

☐ 34. Document ID: US 5663318 A

L3: Entry 34 of 37 File: USPT Sep 2, 1997

US-PAT-NO: 5663318
DOCUMENT-IDENTIFIER: US 5663318 A

TITLE: Assay preparation containing capture and detection polynucleotides covalently

bound to substrates with a heterobifunctional crosslinking agent

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC | Draw Desc | Image

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☐ 35. Document ID: US 5514551 A

L3: Entry 35 of 37 File: USPT May 7, 1996

US-PAT-NO: 5514551
DOCUMENT-IDENTIFIER: US 5514551 A

TITLE: Compositions for the detection of Chlamydia trachomatis

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC | Draw Desc | Image

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☐ 36. Document ID: US 5512445 A

L3: Entry 36 of 37 File: USPT Apr 30, 1996

US-PAT-NO: 5512445
DOCUMENT-IDENTIFIER: US 5512445 A

TITLE: Methods for the detection of Chlamydia trachomatis

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC | Draw Desc | Image

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☐ 37. Document ID: US 5496698 A

L3: Entry 37 of 37 File: USPT Mar 5, 1996

US-PAT-NO: 5496698
DOCUMENT-IDENTIFIER: US 5496698 A

TITLE: Method of isolating ribozyme targets

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC | Draw Desc | Image

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Term	Documents
DETECT$	0
DETECT.DWPI,EPAB,JPAB,USPT,PGPB.	969845
DETECTA.DWPI,EPAB,JPAB,USPT,PGPB.	11
DETECTAB.DWPI,EPAB,JPAB,USPT,PGPB.	1
DETECTABALE.DWPI,EPAB,JPAB,USPT,PGPB.	6
DETECTABCLE.DWPI,EPAB,JPAB,USPT,PGPB.	1
DETECTABE.DWPI,EPAB,JPAB,USPT,PGPB.	7
DETECTABEL.DWPI,EPAB,JPAB,USPT,PGPB.	4
DETECTABIE.DWPI,EPAB,JPAB,USPT,PGPB.	5
DETECTABILI.DWPI,EPAB,JPAB,USPT,PGPB.	4
DETECTABILILTY.DWPI,EPAB,JPAB,USPT,PGPB.	1
(L2 AND (DETECT$ SAME CATALYT$)).USPT,PGPB,JPAB,EPAB,DWPI.	37

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Search Results - Record(s) 11 through 20 of 37 returned.

☐ 11. Document ID: US 6440705 B1

L3: Entry 11 of 37 File: USPT Aug 27, 2002

US-PAT-NO: 6440705
DOCUMENT-IDENTIFIER: US 6440705 B1

TITLE: Method for analyzing polynucleotides

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC | Draw Desc | Image

☐ 12. Document ID: US 6387629 B1

L3: Entry 12 of 37 File: USPT May 14, 2002

US-PAT-NO: 6387629
DOCUMENT-IDENTIFIER: US 6387629 B1

TITLE: Use of cathepsin S in the diagnosis and treatment of endometriosis

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC | Draw Desc | Image

☐ 13. Document ID: US 6361942 B1

L3: Entry 13 of 37 File: USPT Mar 26, 2002

US-PAT-NO: 6361942
DOCUMENT-IDENTIFIER: US 6361942 B1

TITLE: Method, kits and compositions pertaining to detection complexes

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC | Draw Desc | Image

☐ 14. Document ID: US 6358691 B1

L3: Entry 14 of 37 File: USPT Mar 19, 2002

US-PAT-NO: 6358691
DOCUMENT-IDENTIFIER: US 6358691 B1

TITLE: Target-dependent reactions using structure-bridging oligonucleotides

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 15. Document ID: US 6355437 B1

L3: Entry 15 of 37 File: USPT Mar 12, 2002

US-PAT-NO: 6355437
DOCUMENT-IDENTIFIER: US 6355437 B1

TITLE: Target-dependent reactions using structure-bridging oligonucleotides

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 16. Document ID: US 6348314 B1

L3: Entry 16 of 37 File: USPT Feb 19, 2002

US-PAT-NO: 6348314
DOCUMENT-IDENTIFIER: US 6348314 B1

TITLE: Invasive cleavage of nucleic acids

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 17. Document ID: US 6342358 B1

L3: Entry 17 of 37 File: USPT Jan 29, 2002

US-PAT-NO: 6342358
DOCUMENT-IDENTIFIER: US 6342358 B1

TITLE: Human telomerase RNA elements

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 18. Document ID: US 6264825 B1

L3: Entry 18 of 37 File: USPT Jul 24, 2001

US-PAT-NO: 6264825
DOCUMENT-IDENTIFIER: US 6264825 B1

TITLE: Binding acceleration techniques for the detection of analytes

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 19. Document ID: US 6235480 B1

L3: Entry 19 of 37 File: USPT May 22, 2001

US-PAT-NO: 6235480
DOCUMENT-IDENTIFIER: US 6235480 B1

TITLE: Detection of nucleic acid hybrids

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 20. Document ID: US 6225291 B1

L3: Entry 20 of 37 File: USPT May 1, 2001

US-PAT-NO: 6225291
DOCUMENT-IDENTIFIER: US 6225291 B1

TITLE: Rod opsin mRNA-specific ribozyme compositions and methods for the treatment of retinal diseases

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

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Term	Documents
DETECT$	0
DETECT.DWPI,EPAB,JPAB,USPT,PGPB.	969845
DETECTA.DWPI,EPAB,JPAB,USPT,PGPB.	11
DETECTAB.DWPI,EPAB,JPAB,USPT,PGPB.	1
DETECTABALE.DWPI,EPAB,JPAB,USPT,PGPB.	6
DETECTABCLE.DWPI,EPAB,JPAB,USPT,PGPB.	1
DETECTABE.DWPI,EPAB,JPAB,USPT,PGPB.	7
DETECTABEL.DWPI,EPAB,JPAB,USPT,PGPB.	4
DETECTABIE.DWPI,EPAB,JPAB,USPT,PGPB.	5
DETECTABILI.DWPI,EPAB,JPAB,USPT,PGPB.	4
DETECTABILILTY.DWPI,EPAB,JPAB,USPT,PGPB.	1
(L2 AND (DETECT$ SAME CATALYT$)).USPT,PGPB,JPAB,EPAB,DWPI.	37

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Search Results - Record(s) 21 through 30 of 37 returned.

☐ 21. Document ID: US 6218107 B1

L3: Entry 21 of 37 File: USPT Apr 17, 2001

US-PAT-NO: 6218107
DOCUMENT-IDENTIFIER: US 6218107 B1

TITLE: Compositions and methods for detecting the presence of Mycobacterium kansassii

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 22. Document ID: US 6194149 B1

L3: Entry 22 of 37 File: USPT Feb 27, 2001

US-PAT-NO: 6194149
DOCUMENT-IDENTIFIER: US 6194149 B1

TITLE: Target-dependent reactions using structure-bridging oligonucleotides

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 23. Document ID: US 6166178 A

L3: Entry 23 of 37 File: USPT Dec 26, 2000

US-PAT-NO: 6166178
DOCUMENT-IDENTIFIER: US 6166178 A

TITLE: Telomerase catalytic subunit

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 24. Document ID: US 6100027 A

L3: Entry 24 of 37 File: USPT Aug 8, 2000

US-PAT-NO: 6100027
DOCUMENT-IDENTIFIER: US 6100027 A

TITLE: Nucleic acid probes and amplification oligonucleotides for Neisseria species

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 25. Document ID: US 6090606 A

L3: Entry 25 of 37 File: USPT Jul 18, 2000

US-PAT-NO: 6090606
DOCUMENT-IDENTIFIER: US 6090606 A

TITLE: Cleavage agents

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☑ 26. Document ID: US 6090543 A

L3: Entry 26 of 37 File: USPT Jul 18, 2000

US-PAT-NO: 6090543
DOCUMENT-IDENTIFIER: US 6090543 A

TITLE: Cleavage of nucleic acids

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 27. Document ID: US 6074826 A

L3: Entry 27 of 37 File: USPT Jun 13, 2000

US-PAT-NO: 6074826
DOCUMENT-IDENTIFIER: US 6074826 A

TITLE: Nucleic acid amplification oligonucleotides and probes to Lyme disease associated Borrelia

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 28. Document ID: US 6037463 A

L3: Entry 28 of 37 File: USPT Mar 14, 2000

US-PAT-NO: 6037463
DOCUMENT-IDENTIFIER: US 6037463 A

TITLE: Enzymatic RNA molecules that cleave mutant N-RAS

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 29. Document ID: US 6001567 A

L3: Entry 29 of 37 File: USPT Dec 14, 1999

US-PAT-NO: 6001567
DOCUMENT-IDENTIFIER: US 6001567 A

TITLE: Detection of nucleic acid sequences by invader-directed cleavage

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 30. Document ID: US 5994069 A

L3: Entry 30 of 37 File: USPT Nov 30, 1999

US-PAT-NO: 5994069
DOCUMENT-IDENTIFIER: US 5994069 A

TITLE: Detection of nucleic acids by multiple sequential invasive cleavages

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

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Term	Documents
DETECT$	0
DETECT.DWPI,EPAB,JPAB,USPT,PGPB.	969845
DETECTA.DWPI,EPAB,JPAB,USPT,PGPB.	11
DETECTAB.DWPI,EPAB,JPAB,USPT,PGPB.	1
DETECTABALE.DWPI,EPAB,JPAB,USPT,PGPB.	6
DETECTABCLE.DWPI,EPAB,JPAB,USPT,PGPB.	1
DETECTABE.DWPI,EPAB,JPAB,USPT,PGPB.	7
DETECTABEL.DWPI,EPAB,JPAB,USPT,PGPB.	4
DETECTABIE.DWPI,EPAB,JPAB,USPT,PGPB.	5
DETECTABILI.DWPI,EPAB,JPAB,USPT,PGPB.	4
DETECTABILILTY.DWPI,EPAB,JPAB,USPT,PGPB.	1
(L2 AND (DETECT$ SAME CATALYT$)).USPT,PGPB,JPAB,EPAB,DWPI.	37

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L3: Entry 21 of 37 File: USPT Apr 17, 2001

6,218,107

DOCUMENT-IDENTIFIER: US 6218107 B1
TITLE: Compositions and methods for detecting the presence of Mycobacterium kansassii

Detailed Description Text (10):
By "probe" is meant a single-stranded oligonucleotide having a sequence partly or completely complementary to a nucleic acid sequence sought to be detected, so as to stably hybridize thereto under stringent hybridization conditions. In the case of a group or species-specific probe, the probe has the ability to stably hybridize to a target nucleic acid and not to non-target nucleic acids such as those from organisms outside the phylogenetic group or species under stringent hybridization conditions. Probes may, but need not, have regions which are not complementary to a target sequence, as long as such sequences do not substantially alter the probe's desired specificity under stringent hybridization conditions. If such non-complementary regions exist they may contain a 5' promoter sequence and/or a binding site for RNA transcription, a restriction endonuclease recognition site, a non-selective sequence permitting immobilization of the probe or hybridization with a specific second target nucleic acid, or may contain sequences which will confer a desired secondary or tertiary structure, such as a catalytic active site or a hairpin structure on the probe, on the target nucleic acid, or both. A probe may be labeled with a reporter group moiety such as a radioisotope, a fluorescent or chemiluminescent moiety, with an enzyme or other ligand, which can be used for detection or confirmation that the probe has hybridized to the target sequence. One use of a probe is as a hybridization assay probe; probes may also be used as in vivo or in vitro therapeutic oligonucleotides or antisense agents to block or inhibit gene transcription, mRNA splicing, or translation in diseased, infected, or pathogenic cells. -As used in this disclosure, the phrase "a probe (or oligonucleotide) having a nucleic acid sequence consisting essentially of a sequence selected from" a group of specific sequences means that the probe, as a basic and novel characteristic, will form a stable detectable hybrid with a nucleic acid in a nucleotide sequence region having a nucleotide sequence exactly complementary to one of the listed nucleic acid sequences of the group under stringent hybridization conditions. An exact complement under this definition includes the corresponding DNA or RNA sequence.

[redacted]WEST

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L3: Entry 25 of 37 File: USPT Jul 18, 2000

DOCUMENT-IDENTIFIER: US 6090606 A
TITLE: Cleavage agents

Drawing Description Text (24):
FIG. 19A depicts the substrate molecule used to test the ability of synthesis-deficient DNAPs to cleave short hairpin structures.

Drawing Description Text (26):
FIG. 20A shows the A- and I-hairpin molecules used in the trigger/detection assay.

Drawing Description Text (28):
FIG. 20C shows the structure of the cleaved A- and T-hairpin molecules.

Drawing Description Text (29):
FIG. 20D depicts the complementarity between the A- and T-hairpin molecules.

Drawing Description Text (40):
FIG. 30 provides a schematic showing the S-60 hairpin oligonucleotide (SEQ ID NO:40) with the annealed P-15 oligonucletide (SEQ ID NO:41).

Drawing Description Text (41):
FIG. 31 is an autoradiogram of a gel showing the results of a cleavage reaction run using the S-60 hairpin in the presence or absence of the P-15 oligonucleotide.

Drawing Description Text (86):
FIG. 73 is the image generated by a fluorescence imager showing the products produced by the cleavage of the S-60 hairpin using Cleavase.RTM. BN/thrombin (before and after thrombin digestion).

Drawing Description Text (102):
FIGS. 89A-C show the sequence of the S-60 hairpin (SEQ ID NO:40) (A), and the P-15 oligo (SEQ ID NO:41) (shown annealed to the S-60 hairpin in B) and the image generated by a fluorescence imager showing the products produced by cleavage of the S-60 hairpin in the presence of various Invader.TM. oligos.

Detailed Description Text (92):
FIG. 1B provides a schematic of a second embodiment of the detection method of the present invention. Again, the target sequence is recognized by two distinct oligonucleotides in the triggering or trigger reaction and the target nucleic acid aligns the two oligonucleotides for specific cleavage of the 5' arm by the DNAPs of the present invention (not shown in FIG. 1B). The first oligo is completely complementary to a portion of the target sequence. The second oligonucleotide is partially complementary to the target sequence; the 3' end of the second oligonucleotide is fully complementary to the target sequence while the 5' end is non-complementary and forms a single-stranded arm. The non-complementary end of the second oligonucleotide may be a generic sequence which can be used with a set of standard hairpin structures (described below). The detection of different target sequences would require unique portions of two oligonucleotides: the entire first oligonucleotide and the 3' end of the second oligonucleotide. The 5' arm of the second oligonucleotide can be invariant or generic in sequence.

Detailed Description Text (96):

The second part of the detection method allows the annealing of the fragment of the second oligonucleotide liberated by the cleavage of the first cleavage structure formed in the triggering reaction (called the third or trigger oligonucleotide) to a first hairpin structure. This first hairpin structure has a single-stranded 5' arm and a single-stranded 3' arm. The third oligonucleotide triggers the cleavage of this first hairpin structure by annealing to the 3' arm of the hairpin thereby forming a substrate for cleavage by the 5' nuclease of the present invention. The cleavage of this first hairpin structure generates two reaction products: I) the cleaved 5' arm of the hairpin called the fourth oligonucleotide, and 2) the cleaved hairpin structure which now lacks the 5' arm and is smaller in size than the uncleaved hairpin. This cleaved first hairpin may be used as a detection molecule to indicate that cleavage directed by the trigger or third oligonucleotide occurred. Thus, this indicates that the first two oligonucleotides found and annealed to the target sequence thereby indicating the presence of the target sequence in the sample.

Detailed Description Text (97):
The detection products are amplified by having the fourth oligonucleotide anneal to a second hairpin structure. This hairpin structure has a 5'

Detailed Description Text (98):
single-stranded arm and a 3' single-stranded arm. The fourth oligonucleotide generated by cleavage of the first hairpin structure anneals to the 3' arm of the second hairpin structure thereby creating a third cleavage structure recognized by the 5' nuclease. The cleavage of this second hairpin structure also generates two reaction products: I) the cleaved 5' arm of the hairpin called the fifth oligonucleotide which is similar or identical in sequence to the third nucleotide, and 2) the cleaved second hairpin structure which now lacks the 5' arm and is smaller in size than the uncleaved hairpin. This cleaved second hairpin may be as a detection molecule and amplifies the signal generated by the cleavage of the first hairpin structure. Simultaneously with the annealing of the forth oligonucleotide, the third oligonucleotide is dissociated from the cleaved first hairpin molecule so that it is free to anneal to a new copy of the first hairpin structure. The disassociation of the oligonucleotides from the hairpin structures may be accomplished by heating or other means suitable to disrupt base-pairing interactions.

Detailed Description Text (99):
Further amplification of the detection signal is achieved by annealing the fifth oligonucleotide (similar or identical in sequence to the third oligonucleotide) to another molecule of the first hairpin structure. Cleavage is then performed and the oligonucleotide that is liberated then is annealed to another molecule of the second hairpin structure. Successive rounds of annealing and cleavage of the first and second hairpin structures, provided in excess, are performed to generate a sufficient amount of cleaved hairpin products to be detected. The temperature of the detection reaction is cycled just below and just above the annealing temperature for the oligonucleotides used to direct cleavage of the hairpin structures, generally about 55.degree. C. to 70.degree. C. The number of cleavages will double in each cycle until the amount of hairpin structures remaining is below the K.sub.m for the hairpin structures. This point is reached when the hairpin structures are substantially used up. When the detection reaction is to be used in a quantitative manner, the cycling reactions are stopped before the accumulation of the cleaved hairpin detection products reach a plateau.

Detailed Description Text (100):
Detection of the cleaved hairpin structures may be achieved in several ways. In one embodiment detection is achieved by separation on agarose or polyacrylamide gels followed by staining with ethidium bromide. In another embodiment, detection is achieved by separation of the cleaved and uncleaved hairpin structures on a gel followed by autoradiography when the hairpin structures are first labelled with a radioactive probe and separation on chromatography columns using HPLC or FPLC followed by detection of the differently sized fragments by absorption at OD.sub.260. Other means of detection include detection of changes in fluorescence polarization when the single-stranded 5' arm is released by cleavage, the increase in fluorescence of an intercalating fluorescent indicator as the amount of primers

annealed to 3' arms of the hairpin structures increases. The formation of increasing amounts of duplex DNA (between the primer and the 3' arm of the hairpin) occurs if successive rounds of cleavage occur.

Detailed Description Text (101):
The hairpin structures may be attached to a solid support, such as an agarose, styrene or magnetic bead, via the 3' end of the hairpin. A spacer molecule may be placed between the 3' end of the hairpin and the bead, if so desired. The advantage of attaching the hairpin structures to a solid support is that this prevents the hybridization of the two hairpin structures to one another over regions which are complementary. If the hairpin structures anneal to one another, this would reduce the amount of hairpins available for hybridization to the primers released during the cleavage reactions. If the hairpin structures are attached to a solid support, then additional methods of detection of the products of the cleavage reaction may be employed. These methods include, but are not limited to, the measurement of the released single-stranded 5' arm when the 5' arm contains a label at the 5' terminus. This label may be radioactive, fluorescent, biotinylated, etc. If the hairpin structure is not cleaved, the 5' label will remain attached to the solid support. If cleavage occurs, the 5' label will be released from the solid support.

Detailed Description Text (102):
The 3' end of the hairpin molecule may be blocked through the use of dideoxynucleotides. A 3' terminus containing a dideoxynucleotide is unavailable to participate in reactions with certain DNA modifying enzymes, such as terminal transferase. Cleavage of the hairpin having a 3' terminal dideoxynucleotide generates a new, unblocked 3' terminus at the site of cleavage. This new 3' end has a free hydroxyl group which can interact with terminal transferase thus providing another means of detecting the cleavage products.

Detailed Description Text (103):
The hairpin structures are designed so that their self-complementary regions are very short (generally in the range of 3-8 base pairs). Thus, the hairpin structures are not stable at the high temperatures at which this reaction is performed (generally in the range of 50-75.degree. C.) unless the hairpin is stabilized by the presence of the annealed oligonucleotide on the 3' arm of the hairpin. This instability prevents the polymerase from cleaving the hairpin structure in the absence of an associated primer thereby preventing false positive results due to non-oligonucleotide directed cleavage.

Detailed Description Text (104):
As discussed above, the use of the 5' nucleases of the invention which have reduced polymerization activity is advantageous in this method of detecting specific nucleic acid sequences. Significant amounts of polymerization during the cleavage reaction would cause shifting of the site of cleavage in unpredictable ways resulting in the production of a series of cleaved hairpin structures of various sizes rather than a single easily quantifiable product. Additionally, the primers used in one round of cleavage could, if elongated, become unusable for the next cycle, by either forming an incorrect structure or by being too long to melt off under moderate temperature cycling conditions. In a pristine system (i.e., lacking the presence of dNTPs), one could use the unmodified polymerase, but the presence of nucleotides (dNTPs) can decrease the per cycle efficiency enough to give a false negative result. When a crude extract (genomic DNA preparations, crude cell lysates, etc.) is employed or where a sample of DNA from a PCR reaction, or any other sample that might be contaminated with dNTPs, the 5' nucleases of the present invention that were derived from thermostable polymerases are particularly useful.

Detailed Description Text (180):
Some nucleic acid-based detection assays involve the elongation and/or shortening of oligonucleotide probes. For example, as described herein, the primer-directed, primer-independent, and invader-directed cleavage assays, as well as the "nibbling" assay all involve the cleavage (i.e., shortening) of oligonucleotides as a means for detecting the presence of a target nucleic sequence. Examples of other detection assays which involve the shortening of an oligonucleotide probe include the "TaqMan" or nick-translation PCR assay described in U.S. Pat. No. 5,210,015 to Gelfand et al. (the disclosure of which is herein incorporated by reference), the assays described

in U.S. Pat. Nos. 4,775,619 and 5,118,605 to Urdea (the disclosures of which are herein incorporated by reference), the catalytic hybridization amplification assay described in U.S. Pat. No. 5,403,711 to Walder and Walder (the disclosure of which is herein incorporated by reference), and the cycling probe assay described in U.S. Pat. Nos. 4,876,187 and 5,011,769 to Duck et al. (the disclosures of which are herein incorporated by reference). Examples of detection assays which involve the elongation of an oligonucleotide probe (or primer) include the polymerase chain reaction (PCR) described in U.S. Pat. Nos. 4,683,195 and 4,683,202 to Mullis and Mullis et al. (the disclosures of which are herein incorporated by reference) and the ligase chain reaction (LCR) described in U.S. Pat. Nos. 5,427,930 and 5,494,810 to Birkenmeyer et al. and Barany et al. (the disclosures of which are herein incorporated by reference). The above examples are intended to be illustrative of nucleic acid-based detection assays that involve the elongation and/or shortening of oligonucleotide probes and do not provide an exhaustive list.

Detailed Description Text (246):
Testing candidate nucleases for structure-specific activities in these assays is done in much the same way as described for testing modified DNA polymerases in Example 2, but with the use of a different library of model structures. In addition to assessing the enzyme performance in primer-independent and primer-directed cleavage, a set of synthetic hairpins are used to examine the length of duplex downstream of the cleavage site preferred by the enzyme.

Detailed Description Text (247):
The FEN-1 and XPG 5' nucleases used in the present invention must be tested for activity in the assays in which they are intended to be used, including but not limited to the Invader.TM.-directed cleavage detection assay of the present invention and the CFLP.RTM. method of characterizing nucleic acids (the CFLP.RTM. method is described in co-pending application Ser. Nos. 08/337,164, 08/402,601, 08/484,956 and 08/520,946; the disclosures of these applications are incorporated herein by reference). The Invader.TM. assay uses a mode of cleavage that has been termed "primer directed" of "primer dependent" to reflect the influence of the an oligonucleotide hybridized to the target nucleic acid upstream of the cleavage site. In contrast, the CFLP.RTM. reaction is based on the cleavage of folded structure, or hairpins, within the target nucleic acid, in the absence of any hybridized oligonucleotide. The tests described herein are not intended to be limited to the analysis of nucleases with any particular site of cleavage or mode of recognition of substrate structures. It is contemplated that enzymes may be described as 3' nucleases, utilizing the 3' end as a reference point to recognize structures, or may have a yet a different mode of recognition. Further, the use of the term 5' nucleases is not intended to limit consideration to enzymes that cleave the cleavage structures at any particular site. It refers to a general class of enzymes that require some reference or access to a 5' end to effect cleavage of a structure.

Detailed Description Text (249):
The model substrate for testing primer directed cleavage, the "S-60 hairpin" (SEQ ID NO:40) is described in Example 11. In the absence of a primer this hairpin is usually cleaved to release 5' arm fragments of 18 and 19 nucleotides length. An oligonucleotide, termed P-14 (5'-CGAGAGACCACGCT-3' (SEQ ID NO:80)), that extends to the base of the duplex when hybridized to the 3' arm of the S-60 hairpin gives cleavage products of the same size, but at a higher rate of cleavage.

Detailed Description Text (251):
The S-60 hairpin may also be used to test the effects of modifications of the cleavage structure on either primer-directed or invasive cleavage. Such modifications include, but are not limited to, use of mismatches or base analogs in the hairpin duplex at one, a few or all positions, similar disruptions or modifications in the duplex between the primer and the 3' arm of the S-60, chemical or other modifications to one or both ends of the primer sequence, or attachment of moieties to, or other modifications of the 5' arm of the structure. In all of the analyses using the S-60 or a similar hairpin described herein, activity with and without a primer may be compared using the same hairpin structure.

Detailed Description Text (252):
The assembly of these test reactions, including appropriate amounts of hairpin,

primer and candidate nuclease are described in Example 2. As cited therein, the presence of cleavage products is indicated by the presence of molecules which migrate at a lower molecular weight than does the uncleaved test structure. When the reversal of charge of a label is used the products will carry a different net charge than the uncleaved material. Any of these cleavage products indicate that the candidate nuclease has the desired structure-specific nuclease activity. By "desired structure-specific nuclease activity" it is meant only that the candidate nuclease cleaves one or more test molecules. It is not necessary that the candidate nuclease cleave at any particular rate or site of cleavage to be considered successful cleavage.

Detailed Description Text (259):
During the polymerase chain reaction (PCR) [Saiki et al., Science 239:487 (1988); Mullis and Faloona, Methods in Enzymology 155:335 (1987)], DNAPTaq is able to amplify many, but not all, DNA sequences. One sequence that cannot be amplified using DNAPTaq is shown in FIG. 6 (Hairpin structure is SEQ ID NO:15, PRIMERS are SEQ ID NOS:16-17.) This DNA sequence has the distinguishing characteristic of being able to fold on itself to form a hairpin with two single-stranded arms, which correspond to the primers used in PCR.

Detailed Description Text (260):
To test whether this failure to amplify is due to the 5' nuclease activity of the enzyme, we compared the abilities of DNAPTaq and DNAPStf to amplify this DNA sequence during 30 cycles of PCR. Synthetic oligonucleotides were obtained from The Biotechnology Center at the University of Wisconsin-Madison. The DNAPTaq and DNAPStf were from Perkin Elmer (i.e., Amplitaq.TM. DNA polymerase and the Stoffel fragment of Amplitaq.TM. DNA polymerase). The substrate DNA comprised the hairpin structure shown in FIG. 6 cloned in a double-stranded form into pUC19. The primers used in the amplification are listed as SEQ ID NOS:16-17. Primer SEQ ID NO:17 is shown annealed to the 3' arm of the hairpin structure in FIG. 6. Primer SEQ ID NO: 16 is shown as the first 20 nucleotides in bold on the 5' arm of the hairpin in FIG. 6.

Detailed Description Text (263):
To test whether the 5' unpaired nucleotides in the substrate region of this structured DNA are removed by DNAPTaq, the fate of the end-labeled 5' arm during four cycles of PCR was compared using the same two polymerases (FIG. 8). The hairpin templates, such as the one described in FIG. 6, were made using DNAPStf and a .sup.32 P-5'-end-labeled primer. The 5'-end of the DNA was released as a few large fragments by DNAPTaq but not by DNAPStf. The sizes of these fragments (based on their mobilities) show that they contain most or all of the unpaired 5' arm of the DNA. Thus, cleavage occurs at or near the base of the bifurcated duplex. These released fragments terminate with 3' OH groups, as evidenced by direct sequence analysis, and the abilities of the fragments to be extended by terminal deoxynucleotidyl transferase.

Detailed Description Text (264):
FIGS. 9-11 show the results of experiments designed to characterize the cleavage reaction catalyzed by DNAPTaq. Unless otherwise specified, the cleavage reactions comprised 0.01 pmoles of heat-denatured, end-labeled hairpin DNA (with the unlabeled complementary strand also present), 1 pmole primer (complementary to the 3' arm) and 0.5 units of DNAPTaq (estimated to be 0.026 pmoles) in a total volume of 10 .mu.l of 10 mM Tris-Cl, ph 8.5, 50 mM KCl and 1.5 mM MgCl.sub.2. As indicated, some reactions had different concentrations of KCl, and the precise times and temperatures used in each experiment are indicated in the individual figures. The reactions that included a primer used the one shown in FIG. 6 (SEQ ID NO:17). In some instances, the primer was extended to the junction site by providing polymerase and selected nucleotides.

Detailed Description Text (267):
FIG. 9 is an autoradiogram containing the results of a set of experiments and conditions on the cleavage site. FIG. 9A is a determination of reaction components that enable cleavage. Incubation of 5'-end-labeled hairpin DNA was for 30 minutes at 55.degree. C., with the indicated components. The products were resolved by denaturing polyacrylamide gel electrophoresis and the lengths of the products, in nucleotides, are indicated. FIG. 9B describes the effect of temperature on the site of cleavage in the absence of added primer. Reactions were incubated in the absence

of KCl for 10 minutes at the indicated temperatures. The lengths of the products, in nucleotides, are indicated.

Detailed Description Text (269):
Still referring to FIG. 9, the primer is not elongated in the absence of added dNTPs. However, the primer influences both the site and the rate of cleavage of the hairpin. The change in the site of cleavage (FIG. 9A) apparently results from disruption of a short duplex formed between the arms of the DNA substrate. In the absence of primer, the sequences indicated by underlining in FIG. 6 could pair, forming an extended duplex. Cleavage at the end of the extended duplex would release the 11 nucleotide fragment seen on the FIG. 9A lanes with no added primer. Addition of excess primer (FIG. 9A, lanes 3 and 4) or incubation at an elevated temperature (FIG. 9B) disrupts the short extension of the duplex and results in a longer 5' arm and, hence, longer cleavage products.

Detailed Description Text (277):
To determine whether other 5' nucleases in other DNAPs would be suitable for the present invention, an array of enzymes, several of which were reported in the literature to be free of apparent 5' nuclease activity, were examined. The ability of these other enzymes to cleave nucleic acids in a structure-specific manner was tested using the hairpin substrate shown in FIG. 6 under conditions reported to be optimal for synthesis by each enzyme.

Detailed Description Text (284):
The ability of a 5' nuclease to be directed to cleave efficiently at any specific sequence was demonstrated in the following experiment. A partially complementary oligonucleotide termed a "pilot oligonucleotide" was hybridized to sequences at the desired point of cleavage. The non-complementary part of the pilot oligonucleotide provided a structure analogous to the 3' arm of the template (see FIG. 6), whereas the 5' region of the substrate strand became the 5' arm. A primer was provided by designing the 3' region of the pilot so that it would fold on itself creating a short hairpin with a stabilizing tetra-loop [Antao et al., Nucl. Acids Res. 19:5901 (1991)]. Two pilot oligonucleotides are shown in FIG. 12A. Oligonucleotides 19-12 (SEQ ID NO:18), 30-12 (SEQ ID NO:19) and 30-0 (SEQ ID NO:20) are 31, 42 or 30 nucleotides long, respectively. However, oligonucleotides 19-12 (SEQ ID NO:18) and 34-19 (SEQ II) NO:19) have only 19 and 30 nucleotides, respectively, that are complementary to different sequences in the substrate strand. The pilot oligonucleotides are calculated to melt off their complements at about 50.degree. C. (19-12) and about 75.degree. C. (30-12). Both pilots have 12 nucleotides at their 3' ends, which act as 3' arms with base-paired primers attached.

Detailed Description Text (344):
The cleavage structure may be made as a single hairpin molecule, with the 3' end of the target and the 5' end of the pilot joined as a loop as shown in FIG. 16E. A primer oligonucleotide complementary to the 3' arm is also required for these tests so that the enzyme's sensitivity to the presence of a primer may be tested.

Detailed Description Text (345):
Nucleic acids to be used to form test cleavage structures can be chemically synthesized, or can be generated by standard recombinant DNA techniques. By the latter method, the hairpin portion of the molecule can be created by inserting into a cloning vector duplicate copies of a short DNA segment, adjacent to each other but in opposing orientation. The double-stranded fragment encompassing this inverted repeat, and including enough flanking sequence to give short (about 20 nucleotides) unpaired 5' and 3' arms, can then be released from the vector by restriction enzyme digestion, or by PCR performed with an enzyme lacking a 5' exonuclease (e.g., the Stoffel fragment of Amplitaq.TM. DNA polymerase, Vent.TM. DNA polymerase).

Detailed Description Text (346):
The test DNA can be labeled on either end, or internally, with either a radioisotope, or with a non-isotopic tag. Whether the hairpin DNA is a synthetic single strand or a cloned double strand, the DNA is heated prior to use to melt all duplexes. When cooled on ice, the structure depicted in FIG. 16E is formed, and is stable for sufficient time to perform these assays.

Detailed Description Text (347):
To test for primer-directed cleavage (Reaction 1), a detectable quantity of the test molecule (typically 1-100 fmol of .sup.32 P-labeled hairpin molecule) and a 10 to 100-fold molar excess of primer are placed in a buffer known to be compatible with the test enzyme. For Reaction 2, where primer-directed cleavage is performed under condition which allow primer-independent cleavage, the same quantities of molecules are placed in a solution that is the same as the buffer used in Reaction 1 regarding pH, enzyme stabilizers (e.g., bovine serum albumin, nonionic detergents, gelatin) and reducing agents (e.g., dithiothreitol, 2-mercaptoethanol) but that replaces any monovalent cation salt with 20 mM KCl; 20 mM KCl is the demonstrated optimum for primer-independent cleavage. Buffers for enzymes, such as DNAPEc1, that usually operate in the absence of salt are not supplemented to achieve this concentration. To test for primer-independent cleavage (Reaction 3) the same quantity of the test molecule, but no primer, are combined under the same buffer conditions used for Reaction 2.

Detailed Description Text (353):
hairpin substrate molecule shown in FIG. 16E. The substrate molecule was labeled at the 5' terminus with .sup.32 P. 10 fmoles of heat-denatured, end-labeled substrate DNA and 0.5 units of DNAPTaq (lane 1) or 0.5 .mu.l of 4e or 5b extract (FIG. 17, lanes 2-7, extract was prepared as described above) were mixed together in a buffer containing 10 mM Tris-Cl, pH 8.5, 50 mM KCl and 1.5 mM MgCl.sub.2. The final reaction volume was 10 .mu.l. Reactions shown in lanes 4 and 7 contain in addition 50 .mu.M of each dNTP. Reactions shown in lanes 3, 4, 6 and 7 contain 0.2 .mu.M of the primer oligonucleotide (complementary to the 3' arm of the substrate and shown in FIG. 16E). Reactions were incubated at 55.degree. C. for 4 minutes. Reactions were stopped by the addition of 8 .mu.l of 95% formamide containing 20 mM EDTA and 0.05% marker dyes per 10 .mu.l reaction volume. Samples were then applied to 12% denaturing acrylamide gels. Following electrophoresis, the gels were autoradiographed. FIG. 17 shows that clones 4E and 5B exhibit cleavage activity similar to that of the native DNAPTaq. Note that some cleavage occurs in these reactions in the absence of the primer. When long hairpin structure, such as the one used here (FIG. 16E), are used in cleavage reactions performed in buffers containing 50 mM KCl a low level of primer-independent cleavage is seen. Higher concentrations of KCl suppress, but do not eliminate, this primer-independent cleavage under these conditions.

Detailed Description Text (361):
5' Nucleases Derived From Thermostable DNA Polymerases Can Cleave Short Hairpin Structures With Specificity

Detailed Description Text (362):
The ability of the 5' nucleases to cleave hairpin structures to generate a cleaved hairpin structure suitable as a detection molecule was examined. The structure and sequence of the hairpin test molecule is shown in FIG. 19A (SEQ ID NO:15). The oligonucleotide (labeled "primer" in FIG. 19A, SEQ ID NO:22) is shown annealed to its complementary sequence on the 3' arm of the hairpin test molecule. The hairpin test molecule was single-end labeled with .sup.32 P using a labeled T7 promoter primer in a polymerase chain reaction. The label is present on the 5' arm of the hairpin test molecule and is represented by the star in FIG. 19A.

Detailed Description Text (363):
The cleavage reaction was performed by adding 10 fmoles of heat-denatured, end-labeled hairpin test molecule, 0.2 uM of the primer oligonucleotide (complementary to the 3' arm of the hairpin), 50 .mu.M of each dNTP and 0.5 units of DNAPTaq (Perkin Elmer) or 0.5 .mu.l of extract containing a 5' nuclease (prepared as described above) in a total volume of 10 .mu.l in a buffer containing 10 mM Tris-Cl, pH 8.5, 50 mM KCl and 1.5 mM MgCl.sub.2. Reactions shown in lanes 3, 5 and 7 were run in the absence of dNTPs.

Detailed Description Text (364):
Reactions were incubated at 55.degree. C. for 4 minutes. Reactions were stopped at 55.degree. C. by the addition of 8 .mu.l of 95% formamide with 20 mM EDTA and 0.05% marker dyes per 10 .mu.l reaction volume. Samples were not heated before loading onto denaturing polyacrylamide gels (10% polyacrylamide, 19:1 crosslinking, 7 M

urea, 89 mM Tris-borate, pH 8.3, 2.8 mM EDTA). The samples were not heated to allow for the resolution of single-stranded and re-duplexed uncleaved hairpin molecules FIG. 19B shows that altered polymerases lacking any detectable synthetic activity cleave a hairpin structure when an oligonucleotide is annealed to the single-stranded 3' arm of the hairpin to yield a single species of cleaved product (FIG. 19B, lanes 3 and 4). 5' nucleases, such as clone 4D, shown in lanes 3 and 4, produce a single cleaved product even in the presence of dNTPs. 5' nucleases which retain a residual amount of synthetic activity (less than 1% of wild type activity) produce multiple cleavage products as the polymerase can extend the oligonucleotide annealed to the 3' arm of the hairpin thereby moving the site of cleavage (clone 4B, lanes 5 and 6). Native DNATaq produces even more species of cleavage products than do mutant polymerases retaining residual synthetic activity and additionally converts the hairpin structure to a double-stranded form in the presence of dNTPs due to the high level of synthetic activity in the native polymerase (FIG. 19B, lane 8).

Detailed Description Text (367):
To test the ability of an oligonucleotide of the type released in the trigger reaction of the trigger/detection assay to be detected in the detection reaction of the assay, the two hairpin structures shown in FIG. 20A were synthesized using standard techniques. The two hairpins are termed the A-hairpin (SEQ ID NO:23) and the T-hairpin (SEQ ID NO:24). The predicted sites of cleavage in the presence of the appropriate annealed primers are indicated by the arrows. The A- and T-hairpins were designed to prevent intra-strand mis-folding by omitting most of the T residues in the A-hairpin and omitting most of the A residues in the T-hairpin. To avoid mis-priming and slippage, the hairpins were designed with local variations in the sequence motifs (e.g., spacing T residues one or two nucleotides apart or in pairs). The A- and T-hairpins can be annealed together to form a duplex which has appropriate ends for directional cloning in pUC-type vectors; restriction sites are located in the loop regions of the duplex and can be used to elongate the stem regions if desired.

Detailed Description Text (368):
The sequence of the test trigger oligonucleotide is shown in FIG. 20B; this oligonucleotide is termed the alpha primer (SEQ ID NO:25). The alpha primer is complementary to the 3' arm of the T-hairpin as shown in FIG. 20A. When the alpha primer is annealed to the T-hairpin, a cleavage structure is formed that is recognized by thermostable DNA polymerases. Cleavage of the T-hairpin liberates the 5' single-stranded arm of the T-hairpin, generating the tau primer (SEQ ID NO:26) and a cleaved T-hairpin (FIG. 20B; SEQ ID NO:27). The tau primer is complementary to the 3' arm of the A-hairpin as shown in FIG. 20A. Annealing of the tau primer to the A-hairpin generates another cleavage structure; cleavage of this second cleavage structure liberates the 5' single-stranded arm of the A-hairpin, generating another molecule of the alpha primer which then is annealed to another molecule of the T-hairpin. Thermocycling releases the primers so they can function in additional cleavage reactions. Multiple cycles of annealing and cleavage are carried out. The products of the cleavage reactions are primers and the shortened hairpin structures shown in FIG. 20C. The shortened or cleaved hairpin structures may be resolved from the uncleaved hairpins by electrophoresis on denaturing acrylamide gels.

Detailed Description Text (369):
The annealing and cleavage reactions are carried as follows: In a 50 .mu.l reaction volume containing 10 mM Tris-Cl, pH 8.5, 1.0 MgCl.sub.2, 75 mM KCl, 1 pmole of A-hairpin, 1 pmole T-hairpin, the alpha primer is added at equimolar amount relative to the hairpin structures (1 pmole) or at dilutions ranging from 10- to 10.sup.6 -fold and 0.5 .mu.l of extract containing a 5' nuclease (prepared as described above) are added. The predicted melting temperature for the alpha or trigger primer is 60.degree. C. in the above buffer. Annealing is performed just below this predicted melting temperature at 55.degree. C. Using a Perkin Elmer DNA Thermal Cycler, the reactions are annealed at 55.degree. C. for 30 seconds. The temperature is then increased slowly over a five minute period to 72.degree. C. to allow for cleavage. After cleavage, the reactions are rapidly brought to 55.degree. C. (1.degree. C. per second) to allow another cycle of annealing to occur. A range of cycles are performed (20, 40 and 60 cycles) and the reaction products are analyzed at each of these number of cycles. The number of cycles which indicates that the

accumulation of cleaved hairpin products has not reached a plateau is then used for subsequent determinations when it is desirable to obtain a quantitative result.

Detailed Description Text (370):
Following the desired number of cycles, the reactions are stopped at 55.degree. C. by the addition of 8 .mu.l of 95% formamide with 20 mM EDTA and 0.05% marker dyes per 10 .mu.l reaction volume. Samples are not heated before loading onto denaturing polyacrylamide gels (10% polyacrylamide, 19:1 crosslinking, 7 M urea, 89 mM tris-borate, H 8.3, 2.8 mM EDTA). The samples were not heated to allow for the resolution of single-stranded and re-duplexed uncleaved hairpin molecules.

Detailed Description Text (371):
The hairpin molecules may be attached to separate solid support molecules, such as agarose, styrene or magnetic beads, via the 3' end of each hairpin. A spacer molecule may be placed between the 3' end of the hairpin and the bead if so desired. The advantage of attaching the hairpins to a solid support is that this prevents the hybridization of the A- and T-hairpins to one another during the cycles of melting and annealing. The A- and T-hairpins are complementary to one another (as shown in FIG. 20D) and if allowed to anneal to one another over their entire lengths this would reduce the amount of hairpins available for hybridization to the alpha and tau primers during the detection reaction.

Detailed Description Text (372):
The 5' nucleases of the present invention are used in this assay because they lack significant synthetic activity. The lack of synthetic activity results in the production of a single cleaved hairpin product (as shown in FIG. 19B, lane 4). Multiple cleavage products may be generated by 1) the presence of interfering synthetic activity (see FIG. 19B, lanes 6 and 8) or 2) the presence of primer-independent cleavage in the reaction. The presence of primer-independent cleavage is detected in the trigger/detection assay by the presence of different sized products at the fork of the cleavage structure. Primer-independent cleavage can be dampened or repressed, when present, by the use of uncleavable nucleotides in the fork region of the hairpin molecule. For example, thiolated nucleotides can be used to replace several nucleotides at the fork region to prevent primer-independent cleavage.

Detailed Description Text (375):
From the above, it should be clear that native (i.e., "wild type") thermostable DNA polymerases are capable of cleaving hairpin structures in a specific manner and that this discovery can be applied with success to a detection assay. In this example, the mutant DNAPs of the present invention are tested against three different cleavage structures shown in FIG. 22A. Structure 1 in FIG. 22A is simply single stranded 206-mer (the preparation and sequence information for which was discussed above). Structures 2 and 3 are duplexes; structure 2 is the same hairpin structure as shown in FIG. 12A (bottom), while structure 3 has the hairpin portion of structure 2 removed.

Detailed Description Text (419):
FIG. 30 shows a synthetic oligonucleotide which was designed to fold upon itself which consists of the following sequence: 5'-GTTCTCTGCTCTCTGGTCGCTG TCTCGCTTGTGAAACAAGCGAGACAGCGTGGTCTCTCG-3' (SEQ ID ND:40). This oligonucleotide is referred to as the "S-60 Hairpin." The 15 basepair hairpin formed by this oligonucleotide is further stabilized by a "tri-loop" sequence in the loop end (i.e., three nucleotides form the loop portion of the hairpin) [Hiraro, I. et al. (1994) Nucleic Acids Res. 22(4):576]. FIG. 30 also show the sequence of the P-15 oligonucleotide and the location of the region of complementarity shared by the P-15 and S-60 hairpin oligonucleotides. The sequence of the P-15 oligonucleotide is 5'-CGAGAGACCACGCTG-3' (SEQ ID NO:41). As discussed in detail below, the solid black arrowheads shown in FIG. 29 indicate the sites of cleavage of the S-60 hairpin in the absence of the P-15 oligonucleotide and the hollow arrow heads indicate the sites of cleavage in the presence of the P-15 oligonucleotide. The size of the arrow head indicates the relative utilization of a particular site.

Detailed Description Text (420):
The S-60 hairpin molecule was labeled on its 5' end with biotin for subsequent

detection. The S-60 hairpin was incubated in the presence of a thermostable 5' nuclease in the presence or the absence of the P-15 oligonucleotide. The presence of the full duplex which can be formed by the S-60 hairpin is demonstrated by cleavage with the Clevase.RTM. BN 5' nuclease, in a primer-independent fashion (i.e., in the absence of the P-15 oligonucleotide). The release of 18 and 19-nucleotide fragments from the 5' end of the S-60 hairpin molecule showed that the cleavage occurred near the junction between the single and double stranded regions when nothing is hybridized to the 3' arm of the S-60 hairpin (FIG. 31, lane 2).

Detailed Description Text (421):
The reactions shown in FIG. 31 were conducted as follows. Twenty fmole of the 5' biotin-labeled hairpin DNA (SEQ ID NO:40) was combined with 0.1 ng of Cleavase.RTM. BN enzyme and 1 .mu.l of 100 mM MOPS (pH 7.5) containing 0.5% each of Tween-20 and NP-40 in a total volume of 9 .mu.l. In the reaction shown in lane 1, the enzyme was omitted and the volume was made up by addition of distilled water (this served as the uncut or no enzyme control). The reaction shown in lane 3 of FIG. 31 also included 0.5 pmole of the P15 oligonucleotide (SEQ ID NO:41), which can hybridize to the unpaired 3' arm of the S-60 hairpin (SEQ ID NO:40), as diagrammed in FIG. 30.

Detailed Description Text (424):
The resulting autoradiograph is shown in FIG. 31. In FIG. 31, the lane labelled "M" contains the biotinylated P-15 oligonucleotide which served as a marker. The sizes (in nucleotides) of the uncleaved S-60 hairpin (60 nuc; lane 1), the marker (15 nuc; lane "M") and the cleavage products generated by cleavage of the S-60 hairpin in the presence (lane 3) or absence (lane 2) of the P-15 oligonucleotide are indicated.

Detailed Description Text (425):
Because the complementary regions of the S-60 hairpin are located on the same molecule, essentially no lag time should be needed to allow hybridization (i.e., to form the duplex region of the hairpin). This hairpin structure would be expected to form long before the enzyme could locate and cleave the molecule. As expected, cleavage in the absence of the primer oligonucleotide was at or near the junction between the duplex and single-stranded regions, releasing the unpaired 5' arm (FIG. 31, lane 2). The resulting cleavage products were 18 and 19 nucleotides in length.

Detailed Description Text (426):
It was expected that stability of the S-60 hairpin with the tri-loop would prevent the P-15 oligonucleotide from promoting cleavage in the "primer-directed" manner described in Example I above, because the 3' end of the "primer" would remain unpaired. Surprisingly, it was found that the enzyme seemed to mediate an "invasion" by the P-15 primer into the duplex region of the S-60 hairpin, as evidenced by the shifting of the cleavage site 3 to 4 basepairs further into the duplex region, releasing the larger products (22 and 21 nuc.) observed in lane 3 of FIG. 31.

Detailed Description Text (427):
The precise sites of cleavage of the S-60 hairpin are diagrammed on the structure in FIG. 30, with the solid black arrowheads indicating the sites of cleavage in the absence of the P-15 oligonucleotide and the hollow arrow heads indicating the sites of cleavage in the presence of P-15.

Detailed Description Text (428):
These data show that the presence on the 3' arm of an oligonucleotide having some sequence homology with the first several bases of the similarly oriented strand of the downstream duplex can be a dominant factor in determining the site of cleavage by 5' nucleases. Because the oligonucleotide which shares some sequence homology with the first several bases of the similarly oriented strand of the downstream duplex appears to invade the duplex region of the hairpin, it is referred to as an"invader" oligonucleotide. As shown in the examples below, an invader oligonucleotide appears to invade (or displace) a region of duplexed nucleic acid regardless of whether the duplex region is present on the same molecule (i.e., a hairpin) or whether the duplex is formed between two separate nucleic acid strands.

Detailed Description Text (431):
In Example 11 it was demonstrated that an invader oligonucleotide could shift the site at which a 5' nuclease cleaves a duplex region present on a hairpin molecule.

In this example, the ability of an invader oligonucleotide to shift the site of cleavage within a duplex region formed between two separate strands of nucleic acid molecules was examined.

Detailed Description Text (606):
As discussed above in Example 1, the presence of a primer upstream of a bifurcated duplex can influence the site of cleavage, and the existence of a gap between the 3' end of the primer and the base of the duplex can cause a shift of the cleavage site up the unpaired 5' arm of the structure (see also Lyamichev et al., supra and U.S. Pat. No. 5,422,253). The resulting non-invasive shift of the cleavage site in response to a primer is demonstrated in FIGS. 9, 10 and 11, in which the primer used left a 4-nucleotide gap (relative to the base of the duplex). In FIGS. 9-11, all of the "primer-directed" cleavage reactions yielded a 21 nucleotide product, while the primer-independent cleavage reactions yielded a 25 nucleotide product. The site of cleavage obtained when the primer was extended to the base of the duplex, leaving no gap was examined. The results are shown in FIG. 64 (FIG. 64 is a reproduction of FIG. 2C in Lyamichev et al. These data were derived from the cleavage of the structure shown in FIG. 6, as described in Example 1. Unless otherwise specified, the cleavage reactions comprised 0.01 pmoles of heat-denatured, end-labeled hairpin DNA (with the unlabeled complementary strand also present), 1 pmole primer [complementary to the 3' arm shown in FIG. 6 and having the sequence: 5'-GAAT TCGATTTAGGTGACACTATAGAATACA (SEQ ID NO:64)] and 0.5 units of DNAPTaq (estimated to be 0.026 pmoles) in a total volume of 10 .mu.l of 10 mM Tris-Cl, pH 8.5, and 1.5 mM MgCl.sub.2 and 50 mM KCl. The primer was omitted from the reaction shown in the first lane of FIG. 64 and included in lane 2. These reactions were incubated at 55.degree. C. for 10 minutes. Reactions were initiated at the final reaction temperature by the addition of either the MgCl.sub.2 or enzyme. Reactions were stopped at their incubation temperatures by the addition of 8 .mu.l of 95% formamide with 20 mM EDTA and 0.05% marker dyes.

Detailed Description Text (607):
FIG. 64 is an autoradiogram that indicates the effects on the site of cleavage of a bifurcated duplex structure in the presence of a primer that extends to the base of the hairpin duplex. The size of the released cleavage product is shown to the left (i.e., 25 nucleotides). A dideoxynucleotide sequencing ladder of the cleavage substrate is shown on the right as a marker (lanes 3-6).

Detailed Description Text (613):
A comparision between invasive cleavage and primer-directed cleavagem may be illustrated by comparing the expected cleavage sites of a set of probe oligonucleotides having decreasing degrees of complementarity to the target strand in the 5' region of the probe (i.e., the region that overlaps with the invader). A simple test, similar to that performed on the hairpin substrate above (Ex. 25), can be performed to compare invasive cleavage with the non-invasive primer-directed cleavage described above. Such a set of test oligonucleotides is diagrammed in FIG. 65. The structures shown in FIG. 65 are grouped in pairs, labeled "a", "b", "c", and "d". Each pair has the same probe sequence annealed to the target strand (SEQ ID NO:65), but the top structure of each pair is drawn without an upstream oligonucleotide, while the bottom structure includes this oligonucleotide (SEQ ID NO:66). The sequences of the probes shown in FIGS. 64a-64d are listed in SEQ ID NOS:43, 67, 68 and 69, respectively. Probable sites of cleavage are indicated by the black arrowheads. (It is noted that the precise site of cleavage on each of these structures may vary depending on the choice of cleavage agent and other experimental variables. These particular sites are provided for illustrative purposes only.)

Detailed Description Text (629):
To determine the level of hairpin cleavage activity in digested and undigested Cleavase.RTM. BN/thrombin nuclease, dilutions were made and used to cleave a test hairpin containing a 5' fluoroscein label. Varying amounts of digested and undigested Cleavase.RTM. BN/thrombin nuclease were incubated with 5 .mu.M oligonucleotide S-60 hairpin (SEQ ID NO:40; see FIG. 30) in 10 mM MOPS (pH 7.5), 0.05% Tween-20, 0.05% NP-40, and 1 mM MnCl.sub.2 for 5 minutes at 60.degree. C. The digested mixture was electrophoresed on a 20% acrylamide gel and visualized on a Hitachi FMBIO 100 fluoroimager. The resulting image is shown in FIG. 73.

Detailed Description Text (671):
i) Mixed Hairpin Assay

Detailed Description Text (722):
FIGS. 89A and B shows a synthetic oligonucleotide which was designed to fold upon itself which consists of the following sequence: 5'-GTTCTCTGCTCTCT GGTCGCTGTCTCGCTTGTGAAACAAGCGAGACAGCGTGGTCTCTCG-3' (SEQ ID NO:40). This oligonucleotide is referred to as the "S-60 Hairpin." The 15 basepair hairpin formed by this oligonucleotide is further stabilized by a "tri-loop" sequence in the loop end (i.e., three nucleotides form the loop portion of the hairpin) [Hiraro, I. et al. (1994) Nucleic Acids Res. 22(4): 576]. FIG. 89B shows the sequence of the P-15 oligonucleotide (SEQ ID NO:41) and the location of the region of complementarity shared by the P-15 and S-60 hairpin oligonucleotides. In addition to the P-15 oligonucleotide shown, cleavage was also tested in the presence of the P-14 oligonucleotide (SEQ ID NO:122) (P-14 is one base shorter on the 3' end as compared to P-15), the P-14 with an abasic sugar (P-14d; SEQ ID NO:80) and the P14 with an abasic sugar with a 3' phosphate (P-14dp ; SEQ ID NO:81). A P-15 oligo with a 3' phosphate, P-15p (SEQ ID NO:82) was also examined. The black arrows shown in FIG. 89 indicate the sites of cleavage of the S-60 hairpin in the absence (top structure; A) or presence (bottom structure; B) of the P-15 oligonucleotide.

Detailed Description Text (723):
The S-60 hairpin molecule was labeled on its 5' end with fluorescein for subsequent detection. The S-60 hairpin was incubated in the presence of a thermostable 5' nuclease in the presence or the absence of the P-15 oligonucleotide. The presence of the full duplex which can be formed by the S-60 hairpin is demonstrated by cleavage with the Cleavase.RTM. BN 5' nuclease, in a primer-independent fashion (i.e., in the absence of the P-15 oligonucleotide). The release of 18 and 19-nucleotide fragments from the 5' end of the S-60 hairpin molecule showed that the cleavage occurred near the junction between the single and double stranded regions when nothing is hybridized to the 3' arm of the S-60 hairpin (FIG. 31, lane 2).

Detailed Description Text (726):
From the data shown in FIG. 89C, it can be seen that the use of the P-15 Invader.TM. oligonucleotide produces a shift in the cleavage site, while the P14 Invader.TM. oligonucleotide with either a ribose (P14d) or a phosphorylated ribose (P14dp) did not This indicates that the 15th residue of the Invader.TM. oligonucleotide must have the base group attached to promote the shift in cleavage. Interestingly, the addition of phosphate to the 3' end of the P15 oligonucleotide apparently reversed the shifting of cleavage site. The cleavage in this lane may in fact be cleavage in the absence of an Invader.TM. oligonucleotide as is seen in lane 2. In experiments with 5' dye-labeled Invader.TM. oligonucleotides with 3' phosphate groups these oligonucleotides have been severely retarded in gel migration, suggesting that either the enzyme or another constituent of the reaction (e.g., BSA) is able to bind the 3' phosphate irrespective of the rest of the cleavage structure. If the Invader.TM. oligonucleotides are indeed being sequestered away from the cleavage structure, the resulting cleavage of the S-60 hairpin would occur in a "primer-independent" fashion, and would thus not be shifted.

Detailed Description Text (747):
To compare the activity of the Pfu FEN-1 and the Mja FEN-1 nucleases in Invader.TM. reaction the following experiment was performed. A test oligonucleotide IT3 (SEQ ID NO:145) that forms an Invader-Target hairpin structure and probe oligonucleotide PR1 (SEQ ID NO:127) labeled at the 5' end with fluorescein (Integrated DNA Technologies) were employed in Invader.TM. assays using either the Pfu FEN-1 or the Mja FEN-1 nucleases.

Other Reference Publication (29):
Antao et al. "A thermodynamic study of unusually stable RNA and DNA hairpins," Nucl. Acids Res. 19:5901-5905 (1991).

Other Reference Publication (30):
Hiraro et al. "Most compact hairpin-turn structure exerted by a short DNA fragment, d(GCGAAGC) in solution: an extraordinarily stable structure resistant to nucleases and heat," Nuc. Acids Res. 22:576-582 (1994).

L3: Entry 30 of 37 File: USPT Nov 30, 1999

DOCUMENT-IDENTIFIER: US 5994069 A
TITLE: Detection of nucleic acids by multiple sequential invasive cleavages

Brief Summary Text (59):
The invention also provides a method of detecting the presence of a target nucleic acid molecule comprising: a) providing: i) a cleavage means, ii) a source of a target nucleic acid, the target nucleic acid having a first region, a second region and a third region, wherein the first region is located adjacent to and downstream from the second region and wherein the second region is located adjacent to and downstream from the third region; iii) a first oligonucleotide having a 5' and a 3' portion wherein the 5' portion of the first oligonucleotide contains a sequence complementary to (at least a portion of) the second region of the target nucleic acid and wherein the 5' portion of the first oligonucleotide contains a region of self-complementarity and wherein the 3' portion of the first oligonucleotide contains a sequence complementary to (at least a portion of) the third region of the target nucleic acid; iv) a second oligonucleotide having a 5' and a 3' portion wherein the 5' portion of the second oligonucleotide contains a sequence complementary to (at least a portion of) the first region of the target nucleic acid and wherein the 3' portion of the second oligonucleotide contains a sequence complementary to (at least a portion of) the second region of the target nucleic acid; v) a third oligonucleotide having a 5' and a 3' portion wherein the 3' portion of the third oligonucleotide contains a sequence complementary to (at least a portion of) the 5' portion of the first oligonucleotide; b) generating a first cleavage structure wherein at least the 3' portion of the first oligonucleotide is annealed to the target nucleic acid and wherein at least the 5' portion of the second oligonucleotide is annealed to the target nucleic acid and wherein cleavage of the first cleavage structure occurs thereby cleaving the first oligonucleotide to generate a fourth oligonucleotide, the fourth oligonucleotide having a first region, a second region and a third region, wherein the first region is located adjacent to and upstream of the second region and wherein the second region is located adjacent to and upstream of the third region and wherein the third region of the fourth oligonucleotide contains a region of self-complementarity; c) generating a second cleavage structure under conditions wherein the at least the 3' portion of the third oligonucleotide is annealed to the first region of the fourth oligonucleotide and wherein at least the 5' portion of the fourth oligonucleotide is annealed to the second region of the third oligonucleotide and wherein the third region of the fourth oligonucleotide forms a hairpin structure and wherein cleavage of the second cleavage structure occurs to generate a fifth oligonucleotide, the fifth oligonucleotide having a 3'-hydroxyl group; and d) detecting the fifth oligonucleotide.

Drawing Description Text (7):
FIG. 5 depicts a structure which cannot be amplified using DNAPTaq; this figure shows SEQ ID NO:17 (primer) and SEQ ID NO:15 (hairpin).

Drawing Description Text (22):
FIG. 18A depicts the substrate molecule (SEQ ID NOS:15 and 17) used to test the ability of synthesis-deficient DNAPs to cleave short hairpin structures.

Drawing Description Text (32):
FIG. 26 provides a schematic showing the S-60 hairpin oligonucleotide (SEQ ID NO:29) with the annealed P-15 oligonucleotide (SEQ ID NO:30).

Drawing Description Text (33):
FIG. 27 is an autoradiogram of a gel showing the results of a cleavage reaction run using the S-60 hairpin in the presence or absence of the P-I 5 oligonucleotide.

Drawing Description Text (70):
FIG. 62 is the image generated by a fluorescence imager showing the products produced by the cleavage of the S-60 hairpin using Cleavase.RTM. BN/thrombin (before and after thrombin digestion).

Drawing Description Text (86):
FIGS. 78A-C show the sequence of the S-60 hairpin (SEQ ID NO:29) (A), and the P-15 oligo (SEQ ID NO:30) (shown annealed to the S-60 hairpin in B) and the image generated by a fluorescence imager showing the products produced by cleavage of the S-60 hairpin in the presence of various Invader.TM. oligos.

Detailed Description Text (167):
Some nucleic acid-based detection assays involve the elongation and/or shortening of oligonucleotide probes. For example, as described herein, the primer-directed, primer-independent, and invader-directed cleavage assays, as well as the "nibbling" assay all involve the cleavage (i.e., shortening) of oligonucleotides as a means for detecting the presence of a target nucleic sequence. Examples of other detection assays which involve the shortening of an oligonucleotide probe include the "TaqMan" or nick-translation PCR assay described in U.S. Pat. No. 5,210,015 to Gelfand et al. (the disclosure of which is herein incorporated by reference), the assays described in U.S. Pat. Nos. 4,775,619 and 5,118,605 to Urdea (the disclosures of which are herein incorporated by reference), the catalytic hybridization amplification assay described in U.S. Pat. No. 5,403,711 to Walder and Walder (the disclosure of which is herein incorporated by reference), and the cycling probe assay described in U.S. Pat. Nos. 4,876,187 and 5,011,769 to Duck et al. (the disclosures of which are herein incorporated by reference). Examples of detection assays which involve the elongation of an oligonucleotide probe (or primer) include the polymerase chain reaction (PCR) described in U.S. Pat. Nos. 4,683,195 and 4,683,202 to Mullis and Mullis et al. (the disclosures of which are herein incorporated by reference) and the ligase chain reaction (LCR) described in U.S. Pat. Nos. 5,427,930 and 5,494,810 to Birkenmeyer et al. and Barany et al. (the disclosures of which are herein incorporated by reference). The above examples are intended to be illustrative of nucleic acid-based detection assays that involve the elongation and/or shortening of oligonucleotide probes and do not provide an exhaustive list.

Detailed Description Text (231):
Testing candidate nucleases for structure-specific activities in these assays is done in much the same way as described for testing modified DNA polymerases in Example 2, but with the use of a different library of model structures. In addition to assessing the enzyme performance in primer-independent and primer-directed cleavage, a set of synthetic hairpins are used to examine the length of duplex downstream of the cleavage site preferred by the enzyme.

Detailed Description Text (232):
The FEN-1 and XPG 5' nucleases used in the present invention must be tested for activity in the assays in which they are intended to be used, including but not limited to the Invader.TM.-directed cleavage detection assay of the present invention and the CFLP.RTM. method of characterizing nucleic acids (the CFLP.RTM. method is described in co-pending application Ser. Nos. 08/337,164 now abandoned, 08/402,601 now abandoned, 08/484,956 and 08/520,946; the disclosures of these applications are incorporated herein by reference). The Invader.TM. assay uses a mode of cleavage that has been termed "primer directed" of "primer dependent" to reflect the influence of the an oligonucleotide hybridized to the target nucleic acid upstream of the cleavage site. In contrast, the CFLP.RTM. reaction is based on the cleavage of folded structure, or hairpins, within the target nucleic acid, in the absence of any hybridized oligonucleotide. The tests described herein are not intended to be limited to the analysis of nucleases with any particular site of cleavage or mode of recognition of substrate structures. It is contemplated that enzymes may be described as 3' nucleases, utilizing the 3' end as a reference point to recognize structures, or may have a yet a different mode of recognition. Further,

the use of the term 5' nucleases is not intended to limit consideration to enzymes that cleave the cleavage structures at any particular site. It refers to a general class of enzymes that require some reference or access to a 5' end to effect cleavage of a structure.

Detailed Description Text (234):
The model substrate for testing primer directed cleavage, the "S-60 hairpin" (SEQ ID NO:40) is described in Example 11. In the absence of a primer this hairpin is usually cleaved to release 5' arm fragments of 18 and 19 nucleotides length. An oligonucleotide, termed P-14 (5'-CGAGAGACCACGCT-3'; SEQ ID NO:108), that extends to the base of the duplex when hybridized to the 3' arm of the S-60 hairpin gives cleavage products of the same size, but at a higher rate of cleavage.

Detailed Description Text (236):
The S-60 hairpin may also be used to test the effects of modifications of the cleavage structure on either primer-directed or invasive cleavage. Such modifications include, but are not limited to, use of mismatches or base analogs in the hairpin duplex at one, a few or all positions, similar disruptions or modifications in the duplex between the primer and the 3' arm of the S-60, chemical or other modifications to one or both ends of the primer sequence, or attachment of moieties to, or other modifications of the 5' arm of the structure. In all of the analyses using the S-60 or a similar hairpin described herein, activity with and without a primer may be compared using the same hairpin structure.

Detailed Description Text (237):
The assembly of these test reactions, including appropriate amounts of hairpin, primer and candidate nuclease are described in Example 2. As cited therein, the presence of cleavage products is indicated by the presence of molecules which migrate at a lower molecular weight than does the uncleaved test structure. When the reversal of charge of a label is used the products will carry a different net charge than the uncleaved material. Any of these cleavage products indicate that the candidate nuclease has the desired structure-specific nuclease activity. By "desired structure-specific nuclease activity" it is meant only that the candidate nuclease cleaves one or more test molecules. It is not necessary that the candidate nuclease cleave at any particular rate or site of cleavage to be considered successful cleavage.

Detailed Description Text (263):
The product RNA might also be designed to have a catalytic function, e.g., to act as a ribozyme, allowing cleavage another molecule to be indicative of the success of the primary invasive cleavage reaction [Uhlenbeck (1987) Nature 328:596]. In yet another embodiment, the RNA may be made to encode a peptide sequence. When coupled to an in vitro translation system (e.g., the S-30 system derived from E. Coli [Lesley (1985) Methods Mol. Biol. 37:265] or a rabbit reticulocyte lysate system [Dasso and Jackson (1989) Nucleic Acids Res. 17:3129], available from Promega), the production of the appropriate protein may be detected. In a preferred embodiment, the proteins produced include those that allow either calorimetric or luminescent detection, such as beta-galactosidase (lac-Z) or luciferase, respectively.

Detailed Description Text (276):
Another possible configuration for performing a sequential invasive cleavage reaction is diagrammed in FIG. 97. In this case, probe oligonucleotides that are cleaved in the primary reaction can be designed to fold back on themselves (i.e., they contain a region of self-complementarity) to create a molecule that can serve as both the target and Invader.TM. oligonucleotide (termed here an "IT" complex). The IT complex then enables cleavage of a different probe present in the secondary reaction. Inclusion of an excess of the secondary probe molecule ("Probe 2"), allows each IT molecule to serve as the platform for the generation of multiple copies of cleaved secondary probe. In FIG. 97, the regions of self-complementarity contained within the 5' portion of the Invader.TM. oligonucleotide is indicated by the hatched ovals; the arrow between these two ovals indicates that these two regions can self-pair (as shown in the "Cut Probe 1"). The target nucleic acid is divided into three regions based upon which portions of the Invader.TM. and probe oligonucleotides are capable of hybridizing to the target (as discussed above and it is noted that the target may be divided into four regions if a stacker

oligonucleotide is employed). The second cleavage structure is formed by the annealing of the second probe ("Probe 2") to the fragment of Probe 1 ("Cut Probe 1") that was released by cleavage of the first cleavage structure. The Cut Probe 1 forms a hairpin or stem/loop structure near its 3' terminus by virtue of the annealing of the regions of self-complementarity contained within Cut Probe 1 (this self-annealed Cut Probe 1 forms the IT complex). The IT complex (Cut Probe 1) is divided into three regions. Region 1 of the IT complex has complementarity to the 3' portion of Probe 2; region 2 has complementarity to both the 3' end of Cut Probe 1 and to the 5' portion of Probe 2 (analogous to the region of overlap "X" shown in FIG. 25); and region 3 contains the region of self-complementarity (i.e., region 3 is complementary to the 3' portion of the Cut Probe 1). Note that with regard to the IT complex (i.e., Cut Probe 1), region 1 is located upstream of region 2 and region 2 is located upstream of region 3.

Detailed Description Text (285):
Other structure-specific nucleases may be suitable as a well. As discussed in the first example, some 5' nucleases can be used in conditions that significantly reduce this primer -independent cleavage. For example, it has been shown that when the 5' nuclease of DNAPTaq is used to cleave hairpins the primer-independent cleavage is markedly reduced by the inclusion of a monovalent salt in the reaction [Lyamichev, et al. (1993), supra].

Detailed Description Text (287):
A simple test can be performed for any enzyme in combination with any reaction buffer to gauge the amount of Invader.TM. oligonucleotide-independent cleavage to be expected from that combination. A small hairpin-like test molecule that can be used with or without a primer hybridized to a 3' arm, the S-60 molecule, is depicted in FIG. 30. The S-60 and the oligonucleotide P15 are a convenient set of molecules for testing the suitability of an enzyme for application in the present invention and conditions for using these molecules are described in Example 11. Other similar hairpins may be used, of a cleavage structure may be assembled from separate oligonucleotides as diagrammed in FIGS. 99a-e. Reactions using these structures to examine the activity of the Pfu FEN-1 enzyme in the presence or absence of an upstream overlapping oligonucleotide are described in Example 45 and the results are displayed in FIG. 100. To test any particular combination of enzyme and cleavage conditions, similar reactions can be assembled. Outside of the variables of reaction conditions to be tested for any particular enzyme (e.g., salt sensitivities, divalent cation requirements) the test reactions should accommodate any known limitations of the test enzyme. For example, the test reactions should be performed at a temperature that is within the operating temperature range of the candidate enzyme, if known.

Detailed Description Text (289):
If the cleaved product is to serve as an upstream oligonucleotide in a subsequent cleavage reaction, as diagrammed in FIG. 96, the most rapid reaction will be achieved if the other components of the second cleavage structure (i.e., Target 2 and Probe 2 in FIG. 96) are provided in excess so that cleavage may proceed immediately after the upstream oligonucleotide (i.e, Cut Probe 1 in FIG. 96) is made available. To provide an abundance of the second target strand (Target 2 in FIG. 96) one may use an isolated natural nucleic acid, such as bacteriophage M13 DNA, or one may use a synthetic oligonucleotide. If a synthetic oligonucleotide is chosen as the second target sequence, the sequence employed must be examined for regions of self-complementarity (similar considerations apply to short isolated natural nucleic acids such as restriction enzyme fragments or PCR products; natural nucleic acid targets whose 3' end is located >100 nucleotides downstream of the probe binding site on the target strand are sufficiently long enough to obviate the design considerations discussed below). Specifically, it must be determined that the 3' end of the synthetic oligonucleotide may not hybridize to the target strand (i.e., intra-strand hybridization) upstream of the probe, triggering unintended cleavage. Simple examination of the sequence of the synthetic oligonucleotide should reveal if the 3' end has sufficient complementarity to the region of the target upstream of the probe binding site to pose a problem (i.e, it would reveal whether the synthetic oligonucleotide can form a hairpin at its 3' end which could act as an invading oligonucleotide to cause cleavage of the probe in the absence of the hybridization of the intended Invader.TM. oligonucleotide, i.e., the cleavage product from the

first invasive cleavage reaction). If 3 or more of the last 4 to 7 nucleotides (the 3' terminal region) of the synthetic target can basepair upstream of the probe such that there is an invasion into the probe-target duplex, or such that the duplexes formed by the synthetic target strand with its own 3' terminal region and with the probe abut without a gap and the 3' terminal region has an additional 1 or 2 nucleotides unpaired at the extreme 3' end of the synthetic target, then the sequence of the synthetic target oligonucleotide should be modified. The sequence may be changed to disrupt the interaction of the 3' terminal region or to increase the distance between the probe binding site and the regions to which the 3' terminus is binding. Alternatively, the 3' end may be modified to reduce its ability to direct cleavage, e.g., by adding a 3' phosphate during synthesis (see Ex. 35, Table 3) or by adding several additional nucleotides that will not basepair in a self-complementary manner (i.e., they will not participate in the formation of a hairpin structure).

Detailed Description Text (290):
When the product of a first invasive cleavage reaction is designed to form a target which can fold on itself to direct cleavage of a second probe, the IT complex as diagrammed in FIG. 97, the design of the sequence used to form the stem/loop of the IT complex must be considered. To be factored into the design of such a probe are 1) the length of the region of self-complementarity, 2) the length of the region of overlap (region "X" in FIG. 25) and 3) the stability of the hairpin or stem/loop structure as predicted by both Watson-Crick base pairing and by the presence or absence of a particularly stable loop sequence [e.g., a tetraloop (Tinoco et al., supra) or a triloop (Hirao et al., supra)]. It is desirable that this sequence have nucleotides that can base pair (intrastrand), so that the second round of invasive cleavage may occur, but that the structure not be so strong that its presence will prevent the cleavage of the probe in the primary reaction (i.e., Probe 1 in FIG. 96). As shown herein, the presence of a secondary structure in the 5' arm of a cleavage structure cleaved by a structure-specific nuclease may inhibit cleavage by some structure-specific nucleases (Ex. 1).

Detailed Description Text (307):
During the polymerase chain reaction (PCR) [Saiki et al., Science 239:487 (1988); Mullis and Faloona, Methods in Enzymology 155:335 (1987)], DNAPTaq is able to amplify many, but not all, DNA sequences. One sequence that cannot be amplified using DNAPTaq is shown in FIG. 5 (Hairpin structure is SEQ ID NO:15, FIG. 5 also shows a primer: SEQ ID NO:17.) This DNA sequence has the distinguishing characteristic of being able to fold on itself to form a hairpin with two single-stranded arms, which correspond to the primers used in PCR.

Detailed Description Text (308):
To test whether this failure to amplify is due to the 5' nuclease activity of the enzyme, we compared the abilities of DNAPTaq and DNAPStf to amplify this DNA sequence during 30 cycles of PCR. Synthetic oligonucleotides were obtained from The Biotechnology Center at the University of Wisconsin-Madison. The DNAPTaq and DNAPStf were from Perkin Elmer (i.e., Amplitaq.TM. DNA polymerase and the Stoffel fragment of Amplitaq.TM. DNA polymerase). The substrate DNA comprised the hairpin structure shown in FIG. 6 cloned in a double-stranded form into pUC19. The primers used in the amplification are listed as SEQ ID NOS:16-17. Primer SEQ ID NO:17 is shown annealed to the 3' arm of the hairpin structure in FIG. 5. Primer SEQ ID NO: 16 is shown as the first 20 nucleotides in bold on the 5' arm of the hairpin in FIG. 5.

Detailed Description Text (311):
To test whether the 5' unpaired nucleotides in the substrate region of this structured DNA are removed by DNAPTaq, the fate of the end-labeled 5' arm during four cycles of PCR was compared using the same two polymerases (FIG. 7). The hairpin templates, such as the one described in FIG. 5, were made using DNAPStf and a .sup.32 P-5'-end-labeled primer. The 5'-end of the DNA was released as a few large fragments by DNAPTaq but not by DNAPStf. The sizes of these fragments (based on their mobilities) show that they contain most or all of the unpaired 5' arm of the DNA. Thus, cleavage occurs at or near the base of the bifurcated duplex. These released fragments terminate with 3' OH groups, as evidenced by direct sequence analysis, and the abilities of the fragments to be extended by terminal deoxynucleotidyl transferase.

Detailed Description Text (312):
FIGS. 8-10 show the results of experiments designed to characterize the cleavage reaction catalyzed by DNAPTaq. Unless otherwise specified, the cleavage reactions comprised 0.01 pmoles of heat-denatured, end-labeled hairpin DNA (with the unlabeled complementary strand also present), 1 pmole primer (complementary to the 3' arm) and 0.5 units of DNAPTaq (estimated to be 0.026 pmoles) in a total volume of 10 .mu.l of 10 mM Tris-Cl, ph 8.5, 50 mM KCl and 1.5 mM MgCl.sub.2. As indicated, some reactions had different concentrations of KCl, and the precise times and temperatures used in each experiment are indicated in the individual figures. The reactions that included a primer used the one shown in FIG. 5 (SEQ ID NO:17). In some instances, the primer was extended to the junction site by providing polymerase and selected nucleotides.

Detailed Description Text (314):
FIG. 8 is an autoradiogram containing the results of a set of experiments and conditions on the cleavage site. FIG. 8A is a determination of reaction components that enable cleavage. Incubation of 5'-end-labeled hairpin DNA was for 30 minutes at 55.degree. C., with the indicated components. The products were resolved by denaturing polyacrylamide gel electrophoresis and the lengths of the products, in nucleotides, are indicated. FIG. 8B describes the effect of temperature on the site of cleavage in the absence of added primer. Reactions were incubated in the absence of KCl for 10 minutes at the indicated temperatures. The lengths of the products, in nucleotides, are indicated.

Detailed Description Text (316):
Still referring to FIG. 8, the primer is not elongated in the absence of added dNTPs. However, the primer influences both the site and the rate of cleavage of the hairpin. The change in the site of cleavage (FIG. 8A) apparently results from disruption of a short duplex formed between the arms of the DNA substrate. In the absence of primer, the sequences indicated by underlining in FIG. 5 could pair, forming an extended duplex. Cleavage at the end of the extended duplex would release the 11 nucleotide fragment seen on the FIG. 8A lanes with no added primer. Addition of excess primer (FIG. 8A, lanes 3 and 4) or incubation at an elevated temperature (FIG. 8B) disrupts the short extension of the duplex and results in a longer 5' arm and, hence, longer cleavage products.

Detailed Description Text (324):
To determine whether other 5' nucleases in other DNAPs would be suitable for the present invention, an array of enzymes, several of which were reported in the literature to be free of apparent 5' nuclease activity, were examined. The ability of these other enzymes to cleave nucleic acids in a structure-specific manner was tested using the hairpin substrate shown in FIG. 5 under conditions reported to be optimal for synthesis by each enzyme.

Detailed Description Text (331):
The ability of a 5' nuclease to be directed to cleave efficiently at any specific sequence was demonstrated in the following experiment. A partially complementary oligonucleotide termed a "pilot oligonucleotide" was hybridized to sequences at the desired point of cleavage. The non-complementary part of the pilot oligonucleotide provided a structure analogous to the 3' arm of the template (see FIG. 5), whereas the 5' region of the substrate strand became the 5' arm. A primer was provided by designing the 3' region of the pilot so that it would fold on itself creating a short hairpin with a stabilizing tetra-loop [Antao et al., Nucl. Acids Res. 19:5901 (1991)]. Two pilot oligonucleotides are shown in FIG. 11A. Oligonucleotides 19-12 (SEQ ID NO:18), 30-12 (SEQ ID NO:19) and 30-0 (SEQ ID NO:20) are 31, 42 or 30 nucleotides long, respectively. However, oligonucleotides 19-12 (SEQ ID NO:18) and 34-19 (SEQ ID NO:19) have only 19 and 30 nucleotides, respectively, that are complementary to different sequences in the substrate strand. The pilot oligonucleotides are calculated to melt off their complements at about 50.degree. C. (19-12) and about 75.degree. C. (30-12). Both pilots have 12 nucleotides at their 3' ends, which act as 3' arms with base-paired primers attached.

Detailed Description Text (390):
The cleavage structure may be made as a single hairpin molecule, with the 3' end of the target and the 5' end of the pilot joined as a loop as shown in FIG. 15E. A

primer oligonucleotide complementary to the 3' arm is also required for these tests so that the enzyme's sensitivity to the presence of a primer may be tested.

Detailed Description Text (391):
Nucleic acids to be used to form test cleavage structures can be chemically synthesized, or can be generated by standard recombinant DNA techniques. By the latter method, the hairpin portion of the molecule can be created by inserting into a cloning vector duplicate copies of a short DNA segment, adjacent to each other but in opposing orientation. The double-stranded fragment encompassing this inverted repeat, and including enough flanking sequence to give short (about 20 nucleotides) unpaired 5' and 3' arms, can then be released from the vector by restriction enzyme digestion, or by PCR performed with an enzyme lacking a 5' exonuclease (e.g., the Stoffel fragment of Amplitaq.TM. DNA polymerase, Vent.TM. DNA polymerase).

Detailed Description Text (392):
The test DNA can be labeled on either end, or internally, with either a radioisotope, or with a non-isotopic tag. Whether the hairpin DNA is a synthetic single strand or a cloned double strand, the DNA is heated prior to use to melt all duplexes. When cooled on ice, the structure depicted in FIG. 16E is formed, and is stable for sufficient time to perform these assays.

Detailed Description Text (393):
To test for primer-directed cleavage (Reaction 1), a detectable quantity of the test molecule (typically 1-100 fmol of .sup.32 P-labeled hairpin molecule) and a 10 to 100-fold molar excess of primer are placed in a buffer known to be compatible with the test enzyme. For Reaction 2, where primer-directed cleavage is performed under condition which allow primer-independent cleavage, the same quantities of molecules are placed in a solution that is the same as the buffer used in Reaction 1 regarding pH, enzyme stabilizers (e.g., bovine serum albumin, nonionic detergents, gelatin) and reducing agents (e.g., dithiothreitol, 2-mercaptoethanol) but that replaces any monovalent cation salt with 20 mM KCl; 20 mM KCl is the demonstrated optimum for primer-independent cleavage. Buffers for enzymes, such as DNAPEc1, that usually operate in the absence of salt are not supplemented to achieve this concentration. To test for primer-independent cleavage (Reaction 3) the same quantity of the test molecule, but no primer, are combined under the same buffer conditions used for Reaction 2.

Detailed Description Text (399):
For the reactions shown in FIG. 16, the mutant polymerase clones 3E (Taq mutant) and 4B (Tfl mutant) were examined for their ability to cleave the hairpin substrate molecule shown in FIG. 15E. The substrate molecule was labeled at the 5' terminus with .sup.32 P. Ten fmoles of heat-denatured, end-labeled substrate DNA and 0.5 units of DNAPTaq (lane 1) or 0.5 .mu.l of 3E or 4B extract (FIG. 16, lanes 2-7, extract was prepared as described above) were mixed together in a buffer containing 10 mM Tris-Cl, pH 8.5, 50 mM KCl and 1.5 mM MgCl.sub.2. The final reaction volume was 10 .mu.l. Reactions shown in lanes 4 and 7 contain in addition 50 .mu.M of each dNTP. Reactions shown in lanes 3, 4, 6 and 7 contain 0.2 .mu.M of the primer oligonucleotide (complementary to the 3' arm of the substrate and shown in FIG. 15E). Reactions were incubated at 55.degree. C. for 4 minutes. Reactions were stopped by the addition of 8 .mu.l of 95% formamide containing 20 mM EDTA and 0.05% marker dyes per 10 .mu.l reaction volume. Samples were then applied to 12% denaturing acrylamide gels. Following electrophoresis, the gels were autoradiographed. FIG. 16 shows that clones 3E and 4B exhibit cleavage activity similar to that of the native DNAPTaq. Note that some cleavage occurs in these reactions in the absence of the primer. When long hairpin structure, such as the one used here (FIG. 15E), are used in cleavage reactions performed in buffers containing 50 mM KCl a low level of primer-independent cleavage is seen. Higher concentrations of KCl suppress, but do not eliminate, this primer-independent cleavage under these conditions.

Detailed Description Text (407):
5' Nucleases Derived From Thermostable DNA Polymerases Can Cleave Short Hairpin Structures With Specificity

Detailed Description Text (408):

The ability of the 5' nucleases to cleave hairpin structures to generate a cleaved hairpin structure suitable as a detection molecule was examined. The structure and sequence of the hairpin test molecule is shown in FIG. 18A (SEQ ID NO:15). The oligonucleotide (labeled "primer" in FIG. 18A, SEQ ID NO:22) is shown annealed to its complementary sequence on the 3' arm of the hairpin test molecule. The hairpin test molecule was single-end labeled with .sup.32 P using a labeled T7 promoter primer in a polymerase chain reaction. The label is present on the 5' arm of the hairpin test molecule and is represented by the star in FIG. 18A.

Detailed Description Text (409):
The cleavage reaction was performed by adding 10 fmoles of heat-denatured, end-labeled hairpin test molecule, 0.2 .mu.M of the primer oligonucleotide (complementary to the 3' arm of the hairpin), 50 .mu.M of each dNTP and 0.5 units of DNAPTaq (Perkin Elmer) or 0.5 .mu.l of extract containing a 5' nuclease (prepared as described above) in a total volume of 10 .mu.l in a buffer containing 10 mM Tris-Cl, pH 8.5, 50 mM KCl and 1.5 mM MgCl.sub.2. Reactions shown in lanes 3, 5 and 7 were run in the absence of dNTPs.

Detailed Description Text (410):
Reactions were incubated at 55.degree. C. for 4 minutes. Reactions were stopped at 55.degree. C. by the addition of 8 .mu.l of 95% formamide with 20 mM EDTA and 0.05% marker dyes per 10 .mu.l reaction volume. Samples were not heated before loading onto denaturing polyacrylamide gels (10% polyacrylamide, 19:1 crosslinking, 7 M urea, 89 mM Tris-borate, pH 8.3, 2.8 mM EDTA). The samples were not heated to allow for the resolution of single-stranded and re-duplexed uncleaved hairpin molecules.

Detailed Description Text (411):
FIG. 18B shows that altered polymerases lacking any detectable synthetic activity cleave a hairpin structure when an oligonucleotide is annealed to the single-stranded 3' arm of the hairpin to yield a single species of cleaved product (FIG. 18B, lanes 3 and 4). 5' nucleases, such as clone 3D, shown in lanes 3 and 4, produce a single cleaved product even in the presence of dNTPs. 5' nucleases which retain a residual amount of synthetic activity (less than 1% of wild type activity) produce multiple cleavage products as the polymerase can extend the oligonucleotide annealed to the 3' arm of the hairpin thereby moving the site of cleavage (clone 3B, lanes 5 and 6). Native DNATaq produces even more species of cleavage products than do mutant polymerases retaining residual synthetic activity and additionally converts the hairpin structure to a double-stranded form in the presence of dNTPs due to the high level of synthetic activity in the native polymerase (FIG. 18B, lane 8).

Detailed Description Text (414):
From the above, it should be clear that native (i.e., "wild type") thermostable DNA polymerases are capable of cleaving hairpin structures in a specific manner and that this discovery can be applied with success to a detection assay. In this example, the mutant DNAPs of the present invention are tested against three different cleavage structures shown in FIG. 20A. Structure 1 in FIG. 20A is simply single stranded 206-mer (the preparation and sequence information for which was discussed in Example 1C). Structures 2 and 3 are duplexes; structure 2 is the same hairpin structure as shown in FIG. 11A (bottom), while structure 3 has the hairpin portion of structure 2 removed.

Detailed Description Text (451):
FIG. 26 shows a synthetic oligonucleotide which was designed to fold upon itself which consists of the following sequence:
5'-GTTCTCTGCTCTCTGGTCGCTGTCTCGCTTGTGAAACAAGCGAGACAGCGTGGTCTCTCG-3' (SEQ ID NO:29). This oligonucleotide is referred to as the "S-60 Hairpin." The 15 basepair hairpin formed by this oligonucleotide is further stabilized by a "tri-loop" sequence in the loop end (i.e., three nucleotides form the loop portion of the hairpin) [Hiraro, I. et al. (1994) Nucleic Acids Res. 22(4):576]. FIG. 26 also show the sequence of the P-15 oligonucleotide and the location of the region of complementarity shared by the P-15 and S-60 hairpin oligonucleotides. The sequence of the P-15 oligonucleotide is 5'-CGAGAGACCACGCTG-3' (SEQ ID NO:30). As discussed in detail below, the solid black arrowheads shown in FIG. 26 indicate the sites of cleavage of the S-60 hairpin in the absence of the P-15 oligonucleotide and the hollow arrow heads indicate the

sites of cleavage in the presence of the P-15 oligonucleotide. The size of the arrow head indicates the relative utilization of a particular site.

Detailed Description Text (452):
The S-60 hairpin molecule was labeled on its 5' end with biotin for subsequent detection. The S-60 hairpin was incubated in the presence of a thermostable 5' nuclease in the presence or the absence of the P-15 oligonucleotide. The presence of the full duplex which can be formed by the S-60 hairpin is demonstrated by cleavage with the Cleavase.RTM. BN 5' nuclease, in a primer-independent fashion (i.e., in the absence of the P-15 oligonucleotide). The release of 18 and 19-nucleotide fragments from the 5' end of the S-60 hairpin molecule showed that the cleavage occurred near the junction between the single and double stranded regions when nothing is hybridized to the 3' arm of the S-60 hairpin (FIG. 27, lane 2).

Detailed Description Text (453):
The reactions shown in FIG. 27 were conducted as follows. Twenty fmole of the 5' biotin-labeled hairpin DNA (SEQ ID NO:29) was combined with 0.1 ng of Cleavase.RTM. BN enzyme and 1 .mu.l of 100 mM MOPS (pH 7.5) containing 0.5% each of Tween-20 and NP-40 in a total volume of 9 .mu.l. In the reaction shown in lane 1, the enzyme was omitted and the volume was made up by addition of distilled water (this served as the uncut or no enzyme control). The reaction shown in lane 3 of FIG. 27 also included 0.5 pmole of the P15 oligonucleotide (SEQ ID NO:30), which can hybridize to the unpaired 3' arm of the S-60 hairpin (SEQ ID NO:29), as diagrammed in FIG. 26.

Detailed Description Text (456):
The resulting autoradiograph is shown in FIG. 27. In FIG. 27, the lane labelled "M" contains the biotinylated P-15 oligonucleotide which served as a marker. The sizes (in nucleotides) of the uncleaved S-60 hairpin (60 nuc; lane 1), the marker (15 nuc; lane "M") and the cleavage products generated by cleavage of the S-60 hairpin in the presence (lane 3) or absence (lane 2) of the P-15 oligonucleotide are indicated.

Detailed Description Text (457):
Because the complementary regions of the S-60 hairpin are located on the same molecule, essentially no lag time should be needed to allow hybridization (i.e., to form the duplex region of the hairpin). This hairpin structure would be expected to form long before the enzyme could locate and cleave the molecule. As expected, cleavage in the absence of the primer oligonucleotide was at or near the junction between the duplex and single-stranded regions, releasing the unpaired 5' arm (FIG. 27, lane 2). The resulting cleavage products were 18 and 19 nucleotides in length.

Detailed Description Text (458):
It was expected that stability of the S-60 hairpin with the tri-loop would prevent the P-15 oligonucleotide from promoting cleavage in the "primer-directed" manner described in Example 1 above, because the 3' end of the "primer" would remain unpaired. Surprisingly, it was found that the enzyme seemed to mediate an "invasion" by the P-15 primer into the duplex region of the S-60 hairpin, as evidenced by the shifting of the cleavage site 3 to 4 basepairs further into the duplex region, releasing the larger products (22 and 21 nuc.) observed in lane 3 of FIG. 27.

Detailed Description Text (459):
The precise sites of cleavage of the S-60 hairpin are diagrammed on the structure in FIG. 26, with the solid black arrowheads indicating the sites of cleavage in the absence of the P-15 oligonucleotide and the hollow arrow heads indicating the sites of cleavage in the presence of P-15.

Detailed Description Text (460):
These data show that the presence on the 3' arm of an oligonucleotide having some sequence homology with the first several bases of the similarly oriented strand of the downstream duplex can be a dominant factor in determining the site of cleavage by 5' nucleases. Because the oligonucleotide which shares some sequence homology with the first several bases of the similarly oriented strand of the downstream duplex appears to invade the duplex region of the hairpin, it is referred to as an "invader" oligonucleotide. As shown in the examples below, an invader oligonucleotide appears to invade (or displace) a region of duplexed nucleic acid regardless of whether the duplex region is present on the same molecule (i.e., a

hairpin) or whether the duplex is formed between two separate nucleic acid strands.

Detailed Description Text (463):
In Example 10 it was demonstrated that an invader oligonucleotide could shift the site at which a 5' nuclease cleaves a duplex region present on a hairpin molecule. In this example, the ability of an invader oligonucleotide to shift the site of cleavage within a duplex region formed between two separate strands of nucleic acid molecules was examined.

Detailed Description Text (633):
As discussed above in Example 1, the presence of a primer upstream of a bifurcated duplex can influence the site of cleavage, and the existence of a gap between the 3' end of the primer and the base of the duplex can cause a shift of the cleavage site up the unpaired 5' arm of the structure (see also Lyamichev et al., supra and U.S. Pat. No. 5,422,253). The resulting non-invasive shift of the cleavage site in response to a primer is demonstrated in FIGS. 8, 9 and 10, in which the primer used left a 4-nucleotide gap (relative to the base of the duplex). In FIGS. 8-10, all of the "primer-directed" cleavage reactions yielded a 21 nucleotide product, while the primer-independent cleavage reactions yielded a 25 nucleotide product. The site of cleavage obtained when the primer was extended to the base of the duplex, leaving no gap was examined. The results are shown in FIG. 53 (FIG. 53 is a reproduction of FIG. 2C in Lyamichev et al. These data were derived from the cleavage of the structure shown in FIG. 5, as described in Example 1. Unless otherwise specified, the cleavage reactions comprised 0.01 pmoles of heat-denatured, end-labeled hairpin DNA (with the unlabeled complementary strand also present), 1 pmole primer [complementary to the 3' arm shown in FIG. 5 and having the sequence: 5'-GAATTCGATTTAGGTGACACTATAGAATACA (SEQ ID NO:53)] and 0.5 units of DNAPTaq (estimated to be 0.026 pmoles) in a total volume of 10 .mu.l of 10 mM Tris-Cl, pH 8.5, and 1.5 mM MgCl.sub.2 and 50 mM KCl. The primer was omitted from the reaction shown in the first lane of FIG. 53 and included in lane 2. These reactions were incubated at 55.degree. C. for 10 minutes. Reactions were initiated at the final reaction temperature by the addition of either the MgCl.sub.2 or enzyme. Reactions were stopped at their incubation temperatures by the addition of 8 .mu.l of 95% formamide with 20 mM EDTA and 0.05% marker dyes.

Detailed Description Text (634):
FIG. 53 is an autoradiogram that indicates the effects on the site of cleavage of a bifurcated duplex structure in the presence of a primer that extends to the base of the hairpin duplex. The size of the released cleavage product is shown to the left (i.e., 25 nucleotides). A dideoxynucleotide sequencing ladder of the cleavage substrate is shown on the right as a marker (lanes 3-6).

Detailed Description Text (640):
A comparison between invasive cleavage and primer-directed cleavage may be illustrated by comparing the expected cleavage sites of a set of probe oligonucleotides having decreasing degrees of complementarity to the target strand in the 5' region of the probe (i.e., the region that overlaps with the invader). A simple test, similar to that performed on the hairpin substrate above (Ex. 25), can be performed to compare invasive cleavage with the non- invasive primer-directed cleavage described above. Such a set of test oligonucleotides is diagrammed in FIG. 54. The structures shown in FIG. 54 are grouped in pairs, labeled "a", "b", "c", and "d". Each pair has the same probe sequence annealed to the target strand (SEQ ID NO:54), but the top structure of each pair is drawn without an upstream oligonucleotide, while the bottom structure includes this oligonucleotide (SEQ ID NO:55). The sequences of the probes shown in FIGS. 54a-54d are listed in SEQ ID NOS:32, 56, 57 and 58, respectively. Probable sites of cleavage are indicated by the black arrowheads. (It is noted that the precise site of cleavage on each of these structures may vary depending on the choice of cleavage agent and other experimental variables. These particular sites are provided for illustrative purposes only.)

Detailed Description Text (656):
To determine the level of hairpin cleavage activity in digested and undigested Cleavase.RTM. BN/thrombin nuclease, dilutions were made and used to cleave a test hairpin containing a 5' fluoroscein label. Varying amounts of digested and undigested Cleavase.RTM. BN/thrombin nuclease were incubated with 5 .mu.M

oligonucleotide S-60 hairpin (SEQ ID NO:29; see FIG. 26) in 10 mM MOPS (pH 7.5), 0.05% Tween-20, 0.05% NP-40, and 1 mM MnCl.sub.2 for 5 minutes at 60.degree. C. The digested mixture was electrophoresed on a 20% acrylamide gel and visualized on a Hitachi FMBIO 100 fluoroimager. The resulting image is shown in FIG. 62.

Detailed Description Text (696):
i) Mixed Hairpin Assay

Detailed Description Text (745):
FIGS. 78A and B shows a synthetic oligonucleotide which was designed to fold upon itself which consists of the following sequence:
5'-GTTCTCTGCTCTCTGGTCGCTGTCTCGCTTGTGAAACAAGCGAGACAGCGTGGTCTCTCG-3' (SEQ ID NO:29). This oligonucleotide is referred to as the "S-60 Hairpin." The 15 basepair hairpin formed by this oligonucleotide is further stabilized by a "tri-loop" sequence in the loop end (i.e., three nucleotides form the loop portion of the hairpin) [Hiraro, I. et al. (1994) Nucleic Acids Res. 22(4): 576]. FIG. 78B shows the sequence of the P-15 oligonucleotide (SEQ ID NO:30) and the location of the region of complementarity shared by the P-15 and S-60 hairpin oligonucleotides. In addition to the P-15 oligonucleotide shown, cleavage was also tested in the presence of the P-14 oligonucleotide (SEQ ID NO:108) (P-14 is one base shorter on the 3' end as compared to P-15), the P-14 with an abasic sugar (P-14d; SEQ ID NO:109) and the P14 with an abasic sugar with a 3' phosphate (P-14dp; SEQ ID NO:110). A P-15 oligo with a 3' phosphate, P-15p (SEQ ID NO: 111) was also examined. The black arrows shown in FIG. 78 indicate the sites of cleavage of the S-60 hairpin in the absence (top structure; A) or presence (bottom structure; B) of the P-15 oligonucleotide.

Detailed Description Text (746):
The S-60 hairpin molecule was labeled on its 5' end with fluorescein for subsequent detection. The S-60 hairpin was incubated in the presence of a thermostable 5' nuclease in the presence or the absence of the P-15 oligonucleotide. The presence of the full duplex which can be formed by the S-60 hairpin is demonstrated by cleavage with the Cleavase.RTM. BN 5' nuclease, in a primer-independent fashion (i.e., in the absence of the P-15 oligonucleotide). The release of 18 and 19-nucleotide fragments from the 5' end of the S-60 hairpin molecule showed that the cleavage occurred near the junction between the single and double stranded regions when nothing is hybridized to the 3' arm of the S-60 hairpin (FIG. 27, lane 2).

Detailed Description Text (749):
From the data shown in FIG. 78C, it can be seen that the use of the P-15 Invader.TM. oligonucleotide produces a shift in the cleavage site, while the P14 Invader.TM. oligonucleotide with either a ribose (P14d) or a phosphorylated ribose (P14dp) did not This indicates that the 15th residue of the Invader.TM. oligonucleotide must have the base group attached to promote the shift in cleavage. Interestingly, the addition of phosphate to the 3' end of the P15 oligonucleotide apparently reversed the shifting of cleavage site. The cleavage in this lane may in fact be cleavage in the absence of an Invader.TM. oligonucleotide as is seen in lane 2. In experiments with 5' dye-labeled Invader.TM. oligonucleotides with 3' phosphate groups these oligonucleotides have been severely retarded in gel migration, suggesting that either the enzyme or another constituent of the reaction (e.g., BSA) is able to bind the 3' phosphate irrespective of the rest of the cleavage structure. If the Invader.TM. oligonucleotides are indeed being sequestered away from the cleavage structure, the resulting cleavage of the S-60 hairpin would occur in a "primer-independent` fashion, and would thus not be shifted.

Detailed Description Text (769):
To compare the activity of the Pfu FEN-1 and the Mja FEN-1 nucleases in Invader.TM. reaction the following experiment was performed. A test oligonucleotide IT3 (SEQ ID NO:118) that forms an Invader-Target hairpin structure and probe oligonucleotide PR1 (SEQ ID NO:119) labeled at the 5' end with fluorescein (Integrated DNA Technologies) were employed in Invader.TM. assays using either the Pfu FEN-1 or the Mja FEN-1 nucleases.

Other Reference Publication (29):
Antao et al. "A thermodynamic study of unusually stable RNA and DNA hairpins," Nucl. Acids. Res. 19:5901-5905 (1991).

Other Reference Publication (30):
Hiraro et al. "Most compact hairpin-turn structure exerted by a short DNA fragment, d(GCGAAGC) in solution: an extraordinarily stable structure resistant to nucleases and heat," Nuc. Acids Res. 22:576-582 (1994).

CLAIMS:

21. A method of detecting the presence of a target nucleic acid molecule comprising:

a) providing:

i) a cleavage means,

ii) a source of a target nucleic acid, said target nucleic acid having a first region, a second region and a third region, wherein said first region is downstream from said second region and wherein said second region is contiguous to and downstream from said third region;

iii) first and second oligonucleotides having 3' and 5' portions, wherein said 3' portion of said first oligonucleotide contains a sequence complementary to said third region of said target nucleic acid and wherein said 5' portion of said first oligonucleotide and said 3' portion of said second oligonucleotide each contain sequence fully complementary to said second region of said target nucleic acid, and wherein said 5' portion of said second oligonucleotide contains sequence complementary to said first region of said target nucleic acid;

iv) a third oligonucleotide having a 5' and a 3' portion wherein said 3' portion of said third oligonucleotide contains a sequence complementary to said 5' portion of said first oligonucleotide;

b) generating a first cleavage structure wherein at least said 3' portion of said first oligonucleotide is annealed to said target nucleic acid and wherein at least said 5' portion of said second oligonucleotide is annealed to said target nucleic acid and wherein cleavage of said first cleavage structure occurs thereby cleaving said first oligonucleotide to generate a fourth oligonucleotide, said fourth oligonucleotide having a first region, a second region and a third region, wherein said first region is located adjacent to and upstream of said second region and wherein said second region is located adjacent to and upstream of said third region and wherein said third region of said fourth oligonucleotide contains a region of self-complementarity;

c) generating a second cleavage structure under conditions wherein said at least said 3' portion of said third oligonucleotide is annealed to said first region of said fourth oligonucleotide and wherein said third region of said fourth oligonucleotide forms a hairpin structure and wherein cleavage of said second cleavage structure occurs to generate a fifth oligonucleotide, said fifth oligonucleotide having a 3'-hydroxyl group; and

d) detecting said fifth oligonucleotide, thereby detecting the presence of said target nucleic acid.

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TITLE: Invasive cleavage of nucleic acids

Drawing Description Text (24):
FIG. 19A depicts the substrate molecule used to test the ability of synthesis-deficient DNAPs to cleave short hairpin structures.

Drawing Description Text (26):
FIG. 20A shows the A- and T-hairpin molecules used in the trigger/detection assay.

Drawing Description Text (28):
FIG. 20C shows the structure of the cleaved A- and T-hairpin molecules.

Drawing Description Text (29):
FIG. 20D depicts the complementarity between the A- and T-hairpin molecules.

Drawing Description Text (39):
FIG. 30 provides a schematic showing the S-60 hairpin oligonucleotide (SEQ ID NO:40) with the annealed P-15 oligonucletide (SEQ ID NO:41).

Drawing Description Text (40):
FIG. 31 is an autoradiogram of a gel showing the results of a cleavage reaction run using the S-60 hairpin in the presence or absence of the P-15 oligonucleotide.

Detailed Description Text (89):
FIG. 1B provides a schematic of a second embodiment of the detection method of the present invention. Again, the target sequence is recognized by two distinct oligonucleotides in the triggering or trigger reaction and the target nucleic acid aligns the two oligonucleotides for specific cleavage of the 5' arm by the DNAPs of the present invention (not shown in FIG. 1B). The first oligo is completely complementary to a portion of the target sequence. The second oligonucleotide is partially complementary to the target sequence; the 3' end of the second oligonucleotide is fully complementary to the target sequence while the 5' end is non-complementary and forms a single-stranded arm. The non-complementary end of the second oligonucleotide may be a generic sequence which can be used with a set of standard hairpin structures (described below). The detection of different target sequences would require unique portions of two oligonucleotides: the entire first oligonucleotide and the 3' end of the second oligonucleotide. The 5' arm of the second oligonucleotide can be invariant or generic in sequence.

Detailed Description Text (93):
The second part of the detection method allows the annealing of the fragment of the second oligonucleotide liberated by the cleavage of the first cleavage structure formed in the triggering reaction (called the third or trigger oligonucleotide) to a first hairpin structure. This first hairpin structure has a single-stranded 5' arm and a single-stranded 3' arm. The third oligonucleotide triggers the cleavage of this first hairpin structure by annealing to the 3' arm of the hairpin thereby forming a substrate for cleavage by the 5' nuclease of the present invention. The cleavage of this first hairpin structure generates two reaction products: 1) the cleaved 5' arm of the hairpin called the fourth oligonucleotide, and 2) the cleaved hairpin structure which now lacks the 5' arm and is smaller in size than the uncleaved hairpin. This cleaved first hairpin may be used as a detection molecule to

indicate that cleavage directed by the trigger or third oligonucleotide occurred. Thus, this indicates that the first two oligonucleotides found and annealed to the target sequence thereby indicating the presence of the target sequence in the sample.

Detailed Description Text (94):
The detection products are amplified by having the fourth oligonucleotide anneal to a second hairpin structure. This hairpin structure has a 5' single-stranded arm and a 3' single-stranded arm. The fourth oligonucleotide generated by cleavage of the first hairpin structure anneals to the 3' arm of the second hairpin structure thereby creating a third cleavage structure recognized by the 5' nuclease. The cleavage of this second hairpin structure also generates two reaction products: 1) the cleaved 5' arm of the hairpin called the fifth oligonucleotide which is similar or identical in sequence to the third nucleotide, and 2) the cleaved second hairpin structure which now lacks the 5' arm and is smaller in size than the uncleaved hairpin. This cleaved second hairpin may be as a detection molecule and amplifies the signal generated by the cleavage of the first hairpin structure. Simultaneously with the annealing of the forth oligonucleotide, the third oligonucleotide is dissociated from the cleaved first hairpin molecule so that it is free to anneal to a new copy of the first hairpin structure. The disassociation of the oligonucleotides from the hairpin structures may be accomplished by heating or other means suitable to disrupt base-pairing interactions.

Detailed Description Text (95):
Further amplification of the detection signal is achieved by annealing the fifth oligonucleotide (similar or identical in sequence to the third oligonucleotide) to another molecule of the first hairpin structure. Cleavage is then performed and the oligonucleotide that is liberated then is annealed to another molecule of the second hairpin structure. Successive rounds of annealing and cleavage of the first and second hairpin structures, provided in excess, are performed to generate a sufficient amount of cleaved hairpin products to be detected. The temperature of the detection reaction is cycled just below and just above the annealing temperature for the oligonucleotides used to direct cleavage of the hairpin structures, generally about 55.degree. C. to 70.degree. C. The number of cleavages will double in each cycle until the amount of hairpin structures remaining is below the K.sub.m for the hairpin structures. This point is reached when the hairpin structures are substantially used up. When the detection reaction is to be used in a quantitative manner, the cycling reactions are stopped before the accumulation of the cleaved hairpin detection products reach a plateau.

Detailed Description Text (96):
Detection of the cleaved hairpin structures may be achieved in several ways. In one embodiment detection is achieved by separation on agarose or polyacrylamide gels followed by staining with ethidium bromide. In another embodiment, detection is achieved by separation of the cleaved and uncleaved hairpin structures on a gel followed by autoradiography when the hairpin structures are first labelled with a radioactive probe and separation on chromatography columns using HPLC or FPLC followed by detection of the differently sized fragments by absorption at OD.sub.260. Other means of detection include detection of changes in fluorescence polarization when the single-stranded 5' arm is released by cleavage, the increase in fluorescence of an intercalating fluorescent indicator as the amount of primers annealed to 3' arms of the hairpin structures increases. The formation of increasing amounts of duplex DNA (between the primer and the 3' arm of the hairpin) occurs if successive rounds of cleavage occur.

Detailed Description Text (97):
The hairpin structures may be attached to a solid support, such as an agarose, styrene or magnetic bead, via the 3' end of the hairpin. A spacer molecule may be placed between the 3' end of the hairpin and the bead, if so desired. The advantage of attaching the hairpin structures to a solid support is that this prevents the hybridization of the two hairpin structures to one another over regions which are complementary. If the hairpin structures anneal to one another, this would reduce the amount of hairpins available for hybridization to the primers released during the cleavage reactions. If the hairpin structures are attached to a solid support, then additional methods of detection of the products of the cleavage reaction may be

employed. These methods include, but are not limited to, the measurement of the released single-stranded 5' arm when the 5' arm contains a label at the 5' terminus. This label may be radioactive, fluorescent, biotinylated, etc. If the hairpin structure is not cleaved, the 5' label will remain attached to the solid support. If cleavage occurs, the 5' label will be released from the solid support.

Detailed Description Text (98):
The 3' end of the hairpin molecule may be blocked through the use of dideoxynucleotides. A 3' terminus containing a dideoxynucleotide is unavailable to participate in reactions with certain DNA modifying enzymes, such as terminal transferase. Cleavage of the hairpin having a 3' terminal dideoxynucleotide generates a new, unblocked 3' terminus at the site of cleavage. This new 3' end has a free hydroxyl group which can interact with terminal transferase thus providing another means of detecting the cleavage products.

Detailed Description Text (99):
The hairpin structures are designed so that their self-complementary regions are very short (generally in the range of 3-8 base pairs). Thus, the hairpin structures are not stable at the high temperatures at which this reaction is performed (generally in the range of 50-75.degree. C.) unless the hairpin is stabilized by the presence of the annealed oligonucleotide on the 3' arm of the hairpin. This instability prevents the polymerase from cleaving the hairpin structure in the absence of an associated primer thereby preventing false positive results due to non-oligonucleotide directed cleavage.

Detailed Description Text (100):
As discussed above, the use of the 5' nucleases of the invention which have reduced polymerization activity is advantageous in this method of detecting specific nucleic acid sequences. Significant amounts of polymerization during the cleavage reaction would cause shifting of the site of cleavage in unpredictable ways resulting in the production of a series of cleaved hairpin structures of various sizes rather than a single easily quantifiable product. Additionally, the primers used in one round of cleavage could, if elongated, become unusable for the next cycle, by either forming an incorrect structure or by being too long to melt off under moderate temperature cycling conditions. In a pristine system (i.e., lacking the presence of dNTPs), one could use the unmodified polymerase, but the presence of nucleotides (dNTPs) can decrease the per cycle efficiency enough to give a false negative result. When a crude extract (genomic DNA preparations, crude cell lysates, etc.) is employed or where a sample of DNA from a PCR reaction, or any other sample that might be contaminated with dNTPs, the 5' nucleases of the present invention that were derived from thermostable polymerases are particularly useful.

Detailed Description Text (175):
Some nucleic acid-based detection assays involve the elongation and/or shortening of oligonucleotide probes. For example, as described herein, the primer-directed, primer-independent, and invader-directed cleavage assays, as well as the "nibbling" assay all involve the cleavage (i.e., shortening) of oligonucleotides as a means for detecting the presence of a target nucleic sequence. Examples of other detection assays which involve the shortening of an oligonucleotide probe include the "TaqMan" or nick-translation PCR assay described in U.S. Pat. No. 5,210,015 to Gelfand et al. (the disclosure of which is herein incorporated by reference), the assays described in U.S. Pat. Nos. 4,775,619 and 5,118,605 to Urdea (the disclosures of which are herein incorporated by reference), the catalytic hybridization amplification assay described in U.S. Pat. No. 5,403,711 to Walder and Walder (the disclosure of which is herein incorporated by reference), and the cycling probe assay described in U.S. Pat. Nos. 4,876,187 and 5,011,769 to Duck et al. (the disclosures of which are herein incorporated by reference). Examples of detection assays which involve the elongation of an oligonucleotide probe (or primer) include the polymerase chain reaction (PCR) described in U.S. Pat. Nos. 4,683,195 and 4,683,202 to Mullis and Mullis et al. (the disclosures of which are herein incorporated by reference) and the ligase chain reaction (LCR) described in U.S. Pat. Nos. 5,427,930 and 5,494,810 to Birkenmeyer et al. and Barany et al. (the disclosures of which are herein incorporated by reference). The above examples are intended to be illustrative of nucleic acid-based detection assays that involve the elongation and/or shortening of oligonucleotide probes and do not provide an exhaustive list.

Detailed Description Text (238):
Testing candidate nucleases for structure-specific activities in these assays is done in much the same way as described for testing modified DNA polymerases in Example 2, but with the use of a different library of model structures. In addition to assessing the enzyme performance in primer-independent and primer-directed cleavage, a set of synthetic hairpins are used to examine the length of duplex downstream of the cleavage site preferred by the enzyme.

Detailed Description Text (239):
The FEN-1 and XPG 5' nucleases used in the present invention must be tested for activity in the assays in which they are intended to be used, including but not limited to the Invader.TM.-directed cleavage detection assay of the present invention and the CFLP.RTM. method of characterizing nucleic acids (the CFLP.RTM. method is described in co-pending application Ser. Nos. 08/484,956 and 08/520,946and U.S. Pat. Nos. 5,719,028 and 5,843,654; the disclosures of which are incorporated herein by reference). The Invader.TM. assay uses a mode of cleavage that has been termed "primer directed" of "primer dependent" to reflect the influence of the an oligonucleotide hybridized to the target nucleic acid upstream of the cleavage site. In contrast, the CFLP.RTM. reaction is based on the cleavage of folded structure, or hairpins, within the target nucleic acid, in the absence of any hybridized oligonucleotide. The tests described herein are not intended to be limited to the analysis of nucleases with any particular site of cleavage or mode of recognition of substrate structures. It is contemplated that enzymes may be described as 3' nucleases, utilizing the 3' end as a reference point to recognize structures, or may have a yet a different mode of recognition. Further, the use of the term 5' nucleases is not intended to limit consideration to enzymes that cleave the cleavage structures at any particular site. It refers to a general class of enzymes that require some reference or access to a 5' end to effect cleavage of a structure.

Detailed Description Text (241):
The model substrate for testing primer directed cleavage, the "S-60 hairpin" (SEQ ID NO:40) is described in Example 11. In the absence of a primer this hairpin is usually cleaved to release 5' arm fragments of 18 and 19 nucleotides length. An oligonucleotide, termed P-14 (5'-CGAGAGACCACGCT-3'), that extends to the base of the duplex when hybridized to the 3' arm of the S-60 hairpin gives cleavage products of the same size, but at a higher rate of cleavage.

Detailed Description Text (243):
The S-60 hairpin may also be used to test the effects of modifications of the cleavage structure on either primer-directed or invasive cleavage. Such modifications include, but are not limited to, use of mismatches or base analogs in the hairpin duplex at one, a few or all positions, similar disruptions or modifications in the duplex between the primer and the 3' arm of the S-60, chemical or other modifications to one or both ends of the primer sequence, or attachment of moieties to, or other modifications of the 5' arm of the structure. In all of the analyses using the S-60 or a similar hairpin described herein, activity with and without a primer may be compared using the same hairpin structure.

Detailed Description Text (244):
The assembly of these test reactions, including appropriate amounts of hairpin, primer and candidate nuclease are described in Example 2. As cited therein, the presence of cleavage products is indicated by the presence of molecules which migrate at a lower molecular weight than does the uncleaved test structure. When the reversal of charge of a label is used the products will carry a different net charge than the uncleaved material. Any of these cleavage products indicate that the candidate nuclease has the desired structure-specific nuclease activity. By "desired structure-specific nuclease activity" it is meant only that the candidate nuclease cleaves one or more test molecules. It is not necessary that the candidate nuclease cleave at any particular rate or site of cleavage to be considered successful cleavage.

Detailed Description Text (251):
During the polymerase chain reaction (PCR) [Saiki et al., Science 239:487 (1988); Mullis and Faloona, Methods in Enzymology 155:335 (1987)], DNAPTaq is able to

amplify many, but not all, DNA sequences. One sequence that cannot be amplified using DNAPTaq is shown in FIG. 6 (Hairpin structure is SEQ ID NO:15, PRIMERS are SEQ ID NOS:16-17.) This DNA sequence has the distinguishing characteristic of being able to fold on itself to form a hairpin with two single-stranded arms, which correspond to the primers used in PCR.

Detailed Description Text (252):
To test whether this failure to amplify is due to the 5' nuclease activity of the enzyme, we compared the abilities of DNAPTaq and DNAPStf to amplify this DNA sequence during 30 cycles of PCR. Synthetic oligonucleotides were obtained from The Biotechnology Center at the University of Wisconsin-Madison. The DNAPTaq and DNAPStf were from Perkin Elmer (i.e., Amplitaq.TM. DNA polymerase and the Stoffel fragment of Amplitaq.TM. DNA polymerase). The substrate DNA comprised the hairpin structure shown in FIG. 6 cloned in a double-stranded form into pUC 19. The primers used in the amplification are listed as SEQ ID NOS:16-17. Primer SEQ ID NO:17 is shown annealed to the 3' arm of the hairpin structure in FIG. 6. Primer SEQ ID NO:16 is shown as the first 20 nucleotides in bold on the 5' arm of the hairpin in FIG. 6.

Detailed Description Text (255):
To test whether the 5' unpaired nucleotides in the substrate region of this structured DNA are removed by DNAPTaq, the fate of the end-labeled 5' arm during four cycles of PCR was compared using the same two polymerases (FIG. 8). The hairpin templates, such as the one described in FIG. 6, were made using DNAPStf and a .sup.32 P-5'-end-labeled primer. The 5'-end of the DNA was released as a few large fragments by DNAPTaq but not by DNAPStf. The sizes of these fragments (based on their mobilities) show that they contain most or all of the unpaired 5' arm of the DNA. Thus, cleavage occurs at or near the base of the bifurcated duplex. These released fragments terminate with 3' OH groups, as evidenced by direct sequence analysis, and the abilities of the fragments to be extended by terminal deoxynucleotidyl transferase.

Detailed Description Text (256):
FIGS. 9-11 show the results of experiments designed to characterize the cleavage reaction catalyzed by DNAPTaq. Unless otherwise specified, the cleavage reactions comprised 0.01 pmoles of heat-denatured, end-labeled hairpin DNA (with the unlabeled complementary strand also present), 1 pmole primer (complementary to the 3' arm) and 0.5 units of DNAPTaq (estimated to be 0.026 pmoles) in a total volume of 10 .mu.l of 10 mM Tris-Cl, ph 8.5, 50 mM KCl and 1.5 mM MgCl.sub.2. As indicated, some reactions had different concentrations of KCl, and the precise times and temperatures used in each experiment are indicated in the individual figures. The reactions that included a primer used the one shown in FIG. 6 (SEQ ID NO:17). In some instances, the primer was extended to the junction site by providing polymerase and selected nucleotides.

Detailed Description Text (258):
FIG. 9 is an autoradiogram containing the results of a set of experiments and conditions on the cleavage site. FIG. 9A is a determination of reaction components that enable cleavage. Incubation of 5'-end-labeled hairpin DNA was for 30 minutes at 55.degree. C., with the indicated components. The products were resolved by denaturing polyacrylamide gel electrophoresis and the lengths of the products, in nucleotides, are indicated. FIG. 9B describes the effect of temperature on the site of cleavage in the absence of added primer. Reactions were incubated in the absence of KCl for 10 minutes at the indicated temperatures. The lengths of the products, in nucleotides, are indicated.

Detailed Description Text (260):
Still referring to FIG. 9, the primer is not elongated in the absence of added dNTPs. However, the primer influences both the site and the rate of cleavage of the hairpin. The change in the site of cleavage (FIG. 9A) apparently results from disruption of a short duplex formed between the arms of the DNA substrate. In the absence of primer, the sequences indicated by underlining in FIG. 6 could pair, forming an extended duplex. Cleavage at the end of the extended duplex would release the 11 nucleotide fragment seen on the FIG. 9A lanes with no added primer. Addition of excess primer (FIG. 9A, lanes 3 and 4) or incubation at an elevated temperature (FIG. 9B) disrupts the short extension of the duplex and results in a longer 5' arm and, hence, longer cleavage products.

Detailed Description Text (268):
To determine whether other 5' nucleases in other DNAPs would be suitable for the present invention, an array of enzymes, several of which were reported in the literature to be free of apparent 5' nuclease activity, were examined. The ability of these other enzymes to cleave nucleic acids in a structure-specific manner was tested using the hairpin substrate shown in FIG. 6 under conditions reported to be optimal for synthesis by each enzyme.

Detailed Description Text (275):
The ability of a 5' nuclease to be directed to cleave efficiently at any specific sequence was demonstrated in the following experiment. A partially complementary oligonucleotide termed a "pilot oligonucleotide" was hybridized to sequences at the desired point of cleavage. The non-complementary part of the pilot oligonucleotide provided a structure analogous to the 3' arm of the template (see FIG. 6), whereas the 5' region of the substrate strand became the 5' arm. A primer was provided by designing the 3' region of the pilot so that it would fold on itself creating a short hairpin with a stabilizing tetra-loop [Antao et al., Nucl. Acids Res. 19:5901 (1991)]. Two pilot oligonucleotides are shown in FIG. 12A. Oligonucleotides 19-12 (SEQ ID NO:18), 30-12 (SEQ ID NO:19) and 30-0 (SEQ ID NO:20) are 31, 42 or 30 nucleotides long, respectively. However, oligonucleotides 19-12 (SEQ ID NO:18) and 34-19 (SEQ ID NO:19) have only 19 and 30 nucleotides, respectively, that are complementary to different sequences in the substrate strand. The pilot oligonucleotides are calculated to melt off their complements at about 50.degree. C. (19-12) and about 75.degree. C. (30-12). Both pilots have 12 nucleotides at their 3' ends, which act as 3' arms with base-paired primers attached.

Detailed Description Text (334):
The cleavage structure may be made as a single hairpin molecule, with the 3' end of the target and the 5' end of the pilot joined as a loop as shown in FIG. 16E. A primer oligonucleotide complementary to the 3' arm is also required for these tests so that the enzyme's sensitivity to the presence of a primer may be tested.

Detailed Description Text (335):
Nucleic acids to be used to form test cleavage structures can be chemically synthesized, or can be generated by standard recombinant DNA techniques. By the latter method, the hairpin portion of the molecule can be created by inserting into a cloning vector duplicate copies of a short DNA segment, adjacent to each other but in opposing orientation. The double-stranded fragment encompassing this inverted repeat, and including enough flanking sequence to give short (about 20 nucleotides) unpaired 5' and 3' arms, can then be released from the vector by restriction enzyme digestion, or by PCR performed with an enzyme lacking a 5' exonuclease (e.g, the Stoffel fragment of Amplitaq.TM. DNA polymerase, Vent.TM. DNA polymerase).

Detailed Description Text (336):
The test DNA can be labeled on either end, or internally, with either a radioisotope, or with a non-isotopic tag. Whether the hairpin DNA is a synthetic single strand or a cloned double strand, the DNA is heated prior to use to melt all duplexes. When cooled on ice, the structure depicted in FIG. 16E is formed, and is stable for sufficient time to perform these assays.

Detailed Description Text (337):
To test for primer-directed cleavage (Reaction 1), a detectable quantity of the test molecule (typically 1-100 fmol of .sup.32 P-labeled hairpin molecule) and a 10 to 100-fold molar excess of primer are placed in a buffer known to be compatible with the test enzyme. For Reaction 2, where primer-directed cleavage is performed under condition which allow primer-independent cleavage, the same quantities of molecules are placed in a solution that is the same as the buffer used in Reaction 1 regarding pH, enzyme stabilizers (e.g., bovine serum albumin, nonionic detergents, gelatin) and reducing agents (e.g., dithiothreitol, 2-mercaptoethanol) but that replaces any monovalent cation salt with 20 mM KCl; 20 mM KCl is the demonstrated optimum for primer-independent cleavage. Buffers for enzymes, such as DNAPEcl, that usually operate in the absence of salt are not supplemented to achieve this concentration. To test for primer-independent cleavage (Reaction 3) the same quantity of the test molecule, but no primer, are combined under the same buffer conditions used for

Reaction 2.

Detailed Description Text (343):
For the reactions shown in FIG. 17, the mutant polymerase clones 4E (Taq mutant) and 5B (Tfl mutant) were examined for their ability to cleave the hairpin substrate molecule shown in FIG. 16E. The substrate molecule was labeled at the 5' terminus with .sup.32 P. 10 fmoles of heat-denatured, end-labeled substrate DNA and 0.5 units of DNAPTaq (lane 1) or 0.5 .mu.l of 4e or 5b extract (FIG. 17, lanes 2-7, extract was prepared as described above) were mixed together in a buffer containing 10 mM Tris-Cl, pH 8.5, 50 mM KCl and 1.5 mM MgCl.sub.2. The final reaction volume was 10 .mu.l. Reactions shown in lanes 4 and 7 contain in addition 50 .mu.M of each dNTP. Reactions shown in lanes 3, 4, 6 and 7 contain 0.2 .mu.M of the primer oligonucleotide (complementary to the 3' arm of the substrate and shown in FIG. 16E). Reactions were incubated at 55.degree. C. for 4 minutes. Reactions were stopped by the addition of 8 .mu.l of 95% formamide containing 20 mM EDTA and 0.05% marker dyes per 10 .mu.l reaction volume. Samples were then applied to 12% denaturing acrylamide gels. Following electrophoresis, the gels were autoradiographed. FIG. 17 shows that clones 4E and 5B exhibit cleavage activity similar to that of the native DNAPTaq. Note that some cleavage occurs in these reactions in the absence of the primer. When long hairpin structure, such as the one used here (FIG. 16E), are used in cleavage reactions performed in buffers containing 50 mM KCl a low level of primer-independent cleavage is seen. Higher concentrations of KCl suppress, but do not eliminate, this primer-independent cleavage under these conditions.

Detailed Description Text (351):
5' Nucleases Derived from Thermostable DNA Polymerases can Cleave Short Hairpin Structures with Specificity

Detailed Description Text (352):
The ability of the 5' nucleases to cleave hairpin structures to generate a cleaved hairpin structure suitable as a detection molecule was examined. The structure and sequence of the hairpin test molecule is shown in FIG. 19A (SEQ ID NO: 15). The oligonucleotide (labeled "primer" in FIG. 19A, SEQ ID NO:22) is shown annealed to its complementary sequence on the 3' arm of the hairpin test molecule. The hairpin test molecule was single-end labeled with .sup.32 P using a labeled T7 promoter primer in a polymerase chain reaction. The label is present on the 5' arm of the hairpin test molecule and is represented by the star in FIG. 19A.

Detailed Description Text (353):
The cleavage reaction was performed by adding 10 fmoles of heat-denatured, end-labeled hairpin test molecule, 0.2 uM of the primer oligonucleotide (complementary to the 3' arm of the hairpin), 50 .mu.M of each dNTP and 0.5 units of DNAPTaq (Perkin Elmer) or 0.5 .mu.l of extract containing a 5' nuclease (prepared as described above) in a total volume of 10 .mu.l in a buffer containing 10 mM Tris-Cl, pH 8.5, 50 mM KCl and 1.5 mM MgCl.sub.2. Reactions shown in lanes 3, 5 and 7 were run in the absence of dNTPs.

Detailed Description Text (354):
Reactions were incubated at 55.degree. C. for 4 minutes. Reactions were stopped at 55.degree. C. by the addition of 8 .mu.l of 95% formamide with 20 mM EDTA and 0.05% marker dyes per 10 .mu.l reaction volume. Samples were not heated before loading onto denaturing polyacrylamide gels (10% polyacrylamide, 19:1 crosslinking, 7 M urea, 89 mM Tris-borate, pH 8.3, 2.8 mM EDTA). The samples were not heated to allow for the resolution of single-stranded and re-duplexed uncleaved hairpin molecules.

Detailed Description Text (355):
FIG. 19B shows that altered polymerases lacking any detectable synthetic activity cleave a hairpin structure when an oligonucleotide is annealed to the single-stranded 3' arm of the hairpin to yield a single species of cleaved product (FIG. 19B, lanes 3 and 4). 5' nucleases, such as clone 4D, shown in lanes 3 and 4, produce a single cleaved product even in the presence of dNTPs. 5' nucleases which retain a residual amount of synthetic activity (less than 1% of wild type activity) produce multiple cleavage products as the polymerase can extend the oligonucleotide annealed to the 3' arm of the hairpin thereby moving the site of cleavage (clone 4B,

lanes 5 and 6). Native DNATaq produces even more species of cleavage products than do mutant polymerases retaining residual synthetic activity and additionally converts the hairpin structure to a double-stranded form in the presence of dNTPs due to the high level of synthetic activity in the native polymerase (FIG. 19B, lane 8).

Detailed Description Text (358):
To test the ability of an oligonucleotide of the type released in the trigger reaction of the trigger/detection assay to be detected in the detection reaction of the assay, the two hairpin structures shown in FIG. 20A were synthesized using standard techniques. The two hairpins are termed the A-hairpin (SEQ ID NO:23) and the T-hairpin (SEQ ID NO:24). The predicted sites of cleavage in the presence of the appropriate annealed primers are indicated by the arrows. The A- and T-hairpins were designed to prevent intra-strand mis-folding by omitting most of the T residues in the A-hairpin and omitting most of the A residues in the T-hairpin. To avoid mis-priming and slippage, the hairpins were designed with local variations in the sequence motifs (e.g., spacing T residues one or two nucleotides apart or in pairs). The A- and T-hairpins can be annealed together to form a duplex which has appropriate ends for directional cloning in pUC-type vectors; restriction sites are located in the loop regions of the duplex and can be used to elongate the stem regions if desired.

Detailed Description Text (359):
The sequence of the test trigger oligonucleotide is shown in FIG. 20B; this oligonucleotide is termed the alpha primer (SEQ ID NO:25). The alpha primer is complementary to the 3' arm of the T-hairpin as shown in FIG. 20A. When the alpha primer is annealed to the T-hairpin, a cleavage structure is formed that is recognized by thermostable DNA polymerases. Cleavage of the T-hairpin liberates the 5' single-stranded arm of the T-hairpin, generating the tau primer (SEQ ID NO:26) and a cleaved T-hairpin (FIG. 20B; SEQ ID NO:27). The tau primer is complementary to the 3' arm of the A-hairpin as shown in FIG. 20A. Annealing of the tau primer to the A-hairpin generates another cleavage structure; cleavage of this second cleavage structure liberates the 5' single-stranded arm of the A-hairpin, generating another molecule of the alpha primer which then is annealed to another molecule of the T-hairpin. Thermocycling releases the primers so they can function in additional cleavage reactions. Multiple cycles of annealing and cleavage are carried out. The products of the cleavage reactions are primers and the shortened hairpin structures shown in FIG. 20C. The shortened or cleaved hairpin structures may be resolved from the uncleaved hairpins by electrophoresis on denaturing acrylamide gels.

Detailed Description Text (360):
The annealing and cleavage reactions are carried as follows: In a 50 .mu.l reaction volume containing 10 mM Tris-Cl, pH 8.5, 1.0 MgCl.sub.2, 75 mM KCl, 1 pmole of A-hairpin, 1 pmole T-hairpin, the alpha primer is added at equimolar amount relative to the hairpin structures (1 pmole) or at dilutions ranging from 10- to 10.sup.6 -fold and 0.5 .mu.l of extract containing a 5' nuclease (prepared as described above) are added. The predicted melting temperature for the alpha or trigger primer is 60.degree. C. in the above buffer. Annealing is performed just below this predicted melting temperature at 55.degree. C. Using a Perkin Elmer DNA Thermal Cycler, the reactions are annealed at 55.degree. C. for 30 seconds. The temperature is then increased slowly over a five minute period to 72.degree. C. to allow for cleavage. After cleavage, the reactions are rapidly brought to 55.degree. C. (1.degree. C. per second) to allow another cycle of annealing to occur. A range of cycles are performed (20, 40 and 60 cycles) and the reaction products are analyzed at each of these number of cycles. The number of cycles which indicates that the accumulation of cleaved hairpin products has not reached a plateau is then used for subsequent determinations when it is desirable to obtain a quantitative result.

Detailed Description Text (361):
Following the desired number of cycles, the reactions are stopped at 55.degree. C. by the addition of 8 .mu.l of 95% formamide with 20 mM EDTA and 0.05% marker dyes per 10 .mu.l reaction volume. Samples are not heated before loading onto denaturing polyacrylamide gels (10% polyacrylamide, 19:1 crosslinking, 7 M urea, 89 mM tris-borate, pH 8.3, 2.8 mM EDTA). The samples were not heated to allow for the resolution of single-stranded and re-duplexed uncleaved hairpin molecules.

Detailed Description Text (362):
The hairpin molecules may be attached to separate solid support molecules, such as agarose, styrene or magnetic beads, via the 3' end of each hairpin. A spacer molecule may be placed between the 3' end of the hairpin and the bead if so desired. The advantage of attaching the hairpins to a solid support is that this prevents the hybridization of the A- and T-hairpins to one another during the cycles of melting and annealing. The A- and T-hairpins are complementary to one another (as shown in FIG. 20D) and if allowed to anneal to one another over their entire lengths this would reduce the amount of hairpins available for hybridization to the alpha and tau primers during the detection reaction.

Detailed Description Text (363):
The 5' nucleases of the present invention are used in this assay because they lack significant synthetic activity. The lack of synthetic activity results in the production of a single cleaved hairpin product (as shown in FIG. 19B, lane 4). Multiple cleavage products may be generated by 1) the presence of interfering synthetic activity (see FIG. 19B, lanes 6 and 8) or 2) the presence of primer-independent cleavage in the reaction. The presence of primer-independent cleavage is detected in the trigger/detection assay by the presence of different sized products at the fork of the cleavage structure. Primer-independent cleavage can be dampened or repressed, when present, by the use of uncleavable nucleotides in the fork region of the hairpin molecule. For example, thiolated nucleotides can be used to replace several nucleotides at the fork region to prevent primer-independent cleavage.

Detailed Description Text (366):
From the above, it should be clear that native (i.e., "wild type") thermostable DNA polymerases are capable of cleaving hairpin structures in a specific manner and that this discovery can be applied with success to a detection assay. In this example, the mutant DNAPs of the present invention are tested against three different cleavage structures shown in FIG. 22A. Structure 1 in FIG. 22A is simply single stranded 206-mer (the preparation and sequence information for which was discussed above). Structures 2 and 3 are duplexes; structure 2 is the same hairpin structure as shown in FIG. 12A (bottom), while structure 3 has the hairpin portion of structure 2 removed.

Detailed Description Text (409):
FIG. 30 shows a synthetic oligonucleotide which was designed to fold upon itself which consists of the following sequence: 5'-GTTCTCTGCTCTCTGGTCGCTG TCTCGCTTGTGAAACAAGCGAGACAGCGTGGTCTCTCG-3' (SEQ ID NO:40). This oligonucleotide is referred to as the "S-60 Hairpin." The 15 basepair hairpin formed by this oligonucleotide is further stabilized by a "tri-loop" sequence in the loop end (i.e., three nucleotides form the loop portion of the hairpin) [Hiraro, I. et al. (1994) Nucleic Acids Res. 22(4):576]. FIG. 30 also show the sequence of the P-15 oligonucleotide and the location of the region of complementarity shared by the P-15 and S-60 hairpin oligonucleotides. The sequence of the P-15 oligonucleotide is 5'-CGAGAGACCACGCTG-3' (SEQ ID NO:41). As discussed in detail below, the solid black arrowheads shown in FIG. 29 indicate the sites of cleavage of the S-60 hairpin in the absence of the P-15 oligonucleotide and the hollow arrow heads indicate the sites of cleavage in the presence of the P-15 oligonucleotide. The size of the arrow head indicates the relative utilization of a particular site.

Detailed Description Text (410):
The S-60 hairpin molecule was labeled on its 5' end with biotin for subsequent detection. The S-60 hairpin was incubated in the presence of a thermostable 5' nuclease in the presence or the absence of the P-15 oligonucleotide. The presence of the full duplex which can be formed by the S-60 hairpin is demonstrated by cleavage with the CLEAVASE.RTM. BN 5' nuclease, in a primer-independent fashion (i.e., in the absence of the P-15 oligonucleotide). The release of 18 and 19-nucleotide fragments from the 5' end of the S-60 hairpin molecule showed that the cleavage occurred near the junction between the single and double stranded regions when nothing is hybridized to the 3' arm of the S-60 hairpin (FIG. 31, lane 2).

Detailed Description Text (411):

The reactions shown in FIG. 31 were conducted as follows. Twenty fmole of the 5' biotin-labeled hairpin DNA (SEQ ID NO:40) was combined with 0.1 ng of CLEAVASE.RTM. BN enzyme and 1 .mu.l of 100 mM MOPS (pH 7.5) containing 0.5% each of TWEEN-20 and NP-40 in a total volume of 9 .mu.l. In the reaction shown in lane 1, the enzyme was omitted and the volume was made up by addition of distilled water (this served as the uncut or no enzyme control). The reaction shown in lane 3 of FIG. 31 also included 0.5 pmole of the P15 oligonucleotide (SEQ ID NO:41), which can hybridize to the unpaired 3' arm of the S-60 hairpin (SEQ ID NO:40), as diagrammed in FIG. 30.

Detailed Description Text (414):
The resulting autoradiograph is shown in FIG. 31. In FIG. 31, the lane labelled "M" contains the biotinylated P-15 oligonucleotide which served as a marker. The sizes (in nucleotides) of the uncleaved S-60 hairpin (60 nuc; lane 1), the marker (15 nuc; lane "M") and the cleavage products generated by cleavage of the S-60 hairpin in the presence (lane 3) or absence (lane 2) of the P-15 oligonucleotide are indicated.

Detailed Description Text (415):
Because the complementary regions of the S-60 hairpin are located on the same molecule, essentially no lag time should be needed to allow hybridization (i.e., to form the duplex region of the hairpin). This hairpin structure would be expected to form long before the enzyme could locate and cleave the molecule. As expected, cleavage in the absence of the primer oligonucleotide was at or near the junction between the duplex and single-stranded regions, releasing the unpaired 5' arm (FIG. 31, lane 2). The resulting cleavage products were 18 and 19 nucleotides in length.

Detailed Description Text (416):
It was expected that stability of the S-60 hairpin with the tri-loop would prevent the P-15 oligonucleotide from promoting cleavage in the "primer-directed" manner described in Example 1 above, because the 3' end of the "primer" would remain unpaired. Surprisingly, it was found that the enzyme seemed to mediate an "invasion" by the P-15 primer into the duplex region of the S-60 hairpin, as evidenced by the shifting of the cleavage site 3 to 4 basepairs further into the duplex region, releasing the larger products (22 and 21 nuc.) observed in lane 3 of FIG. 31.

Detailed Description Text (417):
The precise sites of cleavage of the S-60 hairpin are diagrammed on the structure in FIG. 30, with the solid black arrowheads indicating the sites of cleavage in the absence of the P-15 oligonucleotide and the hollow arrow heads indicating the sites of cleavage in the presence of P-15.

Detailed Description Text (418):
These data show that the presence on the 3' arm of an oligonucleotide having some sequence homology with the first several bases of the similarly oriented strand of the downstream duplex can be a dominant factor in determining the site of cleavage by 5' nucleases. Because the oligonucleotide which shares some sequence homology with the first several bases of the similarly oriented strand of the downstream duplex appears to invade the duplex region of the hairpin, it is referred to as an "invader" oligonucleotide. As shown in the examples below, an invader oligonucleotide appears to invade (or displace) a region of duplexed nucleic acid regardless of whether the duplex region is present on the same molecule (i.e., a hairpin) or whether the duplex is formed between two separate nucleic acid strands.

Detailed Description Text (421):
In Example 11 it was demonstrated that an invader oligonucleotide could shift the site at which a 5' nuclease cleaves a duplex region present on a hairpin molecule. In this example, the ability of an invader oligonucleotide to shift the site of cleavage within a duplex region formed between two separate strands of nucleic acid molecules was examined.

Detailed Description Text (590):
As discussed above in Example 1, the presence of a primer upstream of a bifurcated duplex can influence the site of cleavage, and the existence of a gap between the 3' end of the primer and the base of the duplex can cause a shift of the cleavage site up the unpaired 5' arm of the structure (see also Lyamichev et al., supra and U.S. Pat. No. 5,422,253). The resulting non-invasive shift of the cleavage site in

response to a primer is demonstrated in FIGS. 9, 10 and 11, in which the primer used left a 4-nucleotide gap (relative to the base of the duplex). In FIGS. 9-11, all of the "primer-directed" cleavage reactions yielded a 21 nucleotide product, while the primer-independent cleavage reactions yielded a 25 nucleotide product. The site of cleavage obtained when the primer was extended to the base of the duplex, leaving no gap was examined. The results are shown in FIG. 64 (FIG. 64 is a reproduction of FIG. 2C in Lyamichev et al. These data were derived from the cleavage of the structure shown in FIG. 6, as described in Example 1. Unless otherwise specified, the cleavage reactions comprised 0.01 pmoles of heat-denatured, end-labeled hairpin DNA (with the unlabeled complementary strand also present), 1 pmole primer [complementary to the 3' arm shown in FIG. 6 and having the sequence: 5'-GAAT TCGATTTAGGTGACACTATAGAATACA (SEQ ID NO:64)] and 0.5 units of DNAPTaq (estimated to be 0.026 pmoles) in a total volume of 10 .mu.l of 10 mM Tris-Cl, pH 8.5, and 1.5 mM MgCl.sub.2 and 50 mM KCl. The primer was omitted from the reaction shown in the first lane of FIG. 64 and included in lane 2. These reactions were incubated at 55.degree. C. for 10 minutes. Reactions were initiated at the final reaction temperature by the addition of either the MgCl.sub.2 or enzyme. Reactions were stopped at their incubation temperatures by the addition of 8 .mu.l of 95% formamide with 20 mM EDTA and 0.05% marker dyes.

Detailed Description Text (591):
FIG. 64 is an autoradiogram that indicates the effects on the site of cleavage of a bifurcated duplex structure in the presence of a primer that extends to the base of the hairpin duplex. The size of the released cleavage product is shown to the left (i.e., 25 nucleotides). A dideoxynucleotide sequencing ladder of the cleavage substrate is shown on the right as a marker (lanes 3-6).

Detailed Description Text (597):
A comparision between invasive cleavage and primer-directed cleavagem may be illustrated by comparing the expected cleavage sites of a set of probe oligonucleotides having decreasing degrees of complementarity to the target strand in the 5' region of the probe (i.e., the region that overlaps with the invader). A simple test, similar to that performed on the hairpin substrate above (Ex. 25), can be performed to compare invasive cleavage with the non- invasive primer-directed cleavage described above. Such a set of test oligonucleotides is diagrammed in FIG. 65. The structures shown in FIG. 65 are grouped in pairs, labeled "a", "b", "c", and "d". Each pair has the same probe sequence annealed to the target strand (SEQ ID NO:65), but the top structure of each pair is drawn without an upstream oligonucleotide, while the bottom structure includes this oligonucleotide (SEQ ID NO:66). The sequences of the probes shown in FIGS. 64a-64d are listed in SEQ ID NOS:43, 67, 68 and 69, respectively. Probable sites of cleavage are indicated by the black arrowheads. (It is noted that the precise site of cleavage on each of these structures may vary depending on the choice of cleavage agent and other experimental variables. These particular sites are provided for illustrative purposes only.)

Other Reference Publication (29):
Antao et al. "A thermodynamic study of unusually stable RNA and DNA hairpins," Nucl. Acids Res., 19:5901-5905 (1991).

Other Reference Publication (30):
Hiraro et al. "Most compact hairpin-turn structure exerted by a short DNA fragment, d(GCGAAGC) in solution: an extraordinarily stable structure resistant to nucleases and heat," Nucl. Acids Res., 22:576-582 (1994).

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L3: Entry 33 of 37 File: USPT May 5, 1998

DOCUMENT-IDENTIFIER: US 5747252 A
TITLE: Nucleic acid probes and amplification oligonucleotides for Neisseria species

Brief Summary Text (36):
By "probe" is meant a single-stranded oligonucleotide having a sequence partly or completely complementary to a nucleic acid sequence sought to be detected, so as to hybridize thereto under stringent hybridization conditions. The term "probe" is meant to exclude nucleic acids normally existing in nature. Purified oligonucleotide probes may be produced by techniques known in the art such as chemical synthesis and by in vitro or in vivo expression from recombinant nucleic acid molecules, e.g., retroviral vectors. Preferably probes are 10 to 100 nucleotides in length. Probes may or may not have regions which are not complementary to a target sequence, so long as such sequences do not substantially affect hybridization under stringent hybridization conditions. If such regions exist they may contain a 5' promoter sequence and/or a binding site for RNA transcription, a restriction endonuclease recognition site, or may contain sequences which will confer a desired secondary or tertiary structure, such as a catalytic active site or a hairpin structure on the probe, on the target nucleic acid, or both. A probe may be labeled with a reporter group moiety such as a radioisotope, a fluorescent or chemiluminescent moiety, with an enzyme or ligand, which can be used for detection or confirmation that the probe has hybridized to the target sequence.

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L3: Entry 34 of 37 File: USPT Sep 2, 1997

DOCUMENT-IDENTIFIER: US 5663318 A
TITLE: Assay preparation containing capture and detection polynucleotides covalently bound to substrates with a heterobifunctional crosslinking agent

Detailed Description Text (11):
It is a particularly preferred embodiment that the free nucleophile is provided as a short sequence of unpaired adenine, guanine, or cytosine (A, G, or C) wherein the short unpaired segment separates paired coding regions of DNA or RNA consisting of a first 5'-3' diagnostic coding sequence, followed by the short unpaired region of A, G, or C, followed by a second 5'-3' coding sequence that is an inverse complement of the first coding sequence; wherein the structure is of a "hairpin" consisting of a single polymer strand paired throughout much of its length; excepting the unpaired sequence of A, G, or C; said unpaired region of A, G, or C free to bind via the exposed amines of the bases to the heterobifunctional crosslinkers described herein, or other crosslinkers known to the art.

Detailed Description Text (13):
It is a particularly preferred embodiment that a capture nucleic acid sequence of the "hairpin" or "handle" type sequence described above be attached to a substrate, such as a dipstick, through the unpaired nucleophilic bearing bases by means of the heterobifunctional reagents herein described, or another means common to the art. It is also a preferred embodiment that a detector probe nucleic acid sequence of the "hairpin" or "handle" structure described above be attached to a plastic bead of less than 1 mm diameter; wherein the unpaired nucleophilic bearing residues are crosslinked to the bead by means of the heterobifunctional reagents herein described, or another means common to the art of nucleophile attachment; and the bead possesses properties such as fluorescence, luminescence, color, radioactivity or magnetism. Further that the capture nucleic acid coated dipstick and the detector nucleic acid coated beads work cooperatively to detect a target sequence of complementary polynucleotide. In still another embodiment both the capture and detector sequence have homology with a target sequence, but neither the capture or detector share any regions of homology with one another. In yet another embodiment of the system the capture and detector probe sequences lack a nucleic acid synthesis polymerase promoting sequence that occurs on the target sequence, wherein the target nucleic acid sequence may be selectively catalytically amplified in the presence of the capture and probe sequences without contamination with unbound capture and probe nucleic acid artifacts.

Detailed Description Text (54):
The sequences each carry an short region hybridizable to the Herpes polymerase gene as well as self-hybridizing into a hairpin loop. This hairpin structure results in an unhybridizing region of amine-beating nucleotides that are not part of the Herpes polymerase gene. The result is a portion of the Herpes gene that can be immobilized via amines on the unhybridized nucleotides.

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L3: Entry 35 of 37 File: USPT May 7, 1996

DOCUMENT-IDENTIFIER: US 5514551 A
TITLE: Compositions for the detection of Chlamydia trachomatis

Brief Summary Text (42):
By "probe" is meant a single-stranded oligonucleotide having a sequence partly or completely complementary to a nucleic acid sequence sought to be detected, so as to stably hybridize thereto under stringent hybridization conditions. In the case of a group or species-specific probe, the probe has the ability to stably hybridize to a target nucleic acid and not to non-target nucleic acids such as those from organisms outside the phylogenetic group or species under stringent hybridization conditions. Probes may, but need not, have regions which are not complementary to a target sequence, as long as such sequences do not substantially alter the probe's desired specificity under stringent hybridization conditions. If such non-complementary regions exist they may contain a 5' promoter sequence and/or a binding site for RNA transcription, a restriction endonuclease recognition site, or may contain sequences which will confer a desired secondary or tertiary structure, such as a catalytic active site or a hairpin structure on the probe, on the target nucleic acid, or both. A probe may be labeled with a reporter group moiety such as a radioisotope, a fluorescent or chemiluminescent moiety, with an enzyme or other ligand, which can be used for detection or confirmation that the probe has hybridized to the target sequence. One use of a probe is as a hybridization assay probe; probes may also be used as in vivo or in vitro therapeutic oligonucleotides or antisense agents to block or inhibit gene transcription, mRNA splicing, or translation in diseased, infected, or pathogenic cells.

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L3: Entry 36 of 37 File: USPT Apr 30, 1996

DOCUMENT-IDENTIFIER: US 5512445 A
TITLE: Methods for the detection of Chlamydia trachomatis

Brief Summary Text (43):
By "probe" is meant a single-stranded oligonucleotide having a sequence partly or completely complementary to a nucleic acid sequence sought to be detected, so as to stably hybridize thereto under stringent hybridization conditions. In the case of a group or species-specific probe, the probe has the ability to stably hybridize to a target nucleic acid and not to non-target nucleic acids such as those from organisms outside the phylogenetic group or species under stringent hybridization conditions. Probes may, but need not, have regions which are not complementary to a target sequence, as long as such sequences do not substantially alter the probe's desired specificity under stringent hybridization conditions. If such non-complementary regions exist they may contain a 5' promoter sequence and/or a binding site for RNA transcription, a restriction endonuclease recognition site, or may contain sequences which will confer a desired secondary or tertiary structure, such as a catalytic active site or a hairpin structure on the probe, on the target nucleic acid, or both. A probe may be labeled with a reporter group moiety such as a radioisotope, a fluorescent or chemiluminescent moiety, with an enzyme or other ligand, which can be used for detection or confirmation that the probe has hybridized to the target sequence. One use of a probe is as a hybridization assay probe; probes may also be used as in vivo or in vitro therapeutic oligonucleotides or antisense agents to block or inhibit gene transcription, mRNA splicing, or translation in diseased, infected, or pathogenic cells.

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L3: Entry 27 of 37 File: USPT Jun 13, 2000

DOCUMENT-IDENTIFIER: US 6074826 A
TITLE: Nucleic acid amplification oligonucleotides and probes to Lyme disease associated Borrelia

Brief Summary Text (121):
By "probe" is meant a single-stranded oligonucleotide having a sequence partly or completely complementary to a nucleic acid sequence sought to be detected, so as to hybridize thereto under stringent hybridization conditions. The term "probe" is meant to exclude naturally occurring nucleic acids. Purified oligonucleotide probes may be produced by techniques known in the art such as chemical synthesis and by in vitro or in vivo expression from recombinant nucleic acid molecules, e.g., retroviral vectors. Preferably probes are 10 to 100 nucleotides in length. Probes may or may not have regions which are not complementary to a target sequence, so long as such sequences do not substantially affect hybridization under stringent hybridization conditions. If such regions exist they may contain a 5' promoter sequence and/or a binding site for RNA transcription, a restriction endonuclease recognition site, or may contain sequences which will confer a desired secondary or tertiary structure, such as a catalytic active site or a hairpin structure on the probe, on the target nucleic acid, or both. A probe may be labeled with a reporter group moiety such as a radioisotope, a fluorescent or chemiluminescent moiety, with an enzyme or other ligand, which can be used for detection or confirmation that the probe has hybridized to the target sequence.

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*File 155: Alert feature enhanced for multiple files, duplicates
removal, customized scheduling. See HELP ALERT.
  File   5:Biosis Previews(R)  1969-2002/Oct W2
         (c) 2002 BIOSIS
*File   5: Alert feature enhanced for multiple files, duplicates
removal, customized scheduling. See HELP ALERT.

      Set  Items  Description
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          422946  TARGET?
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? s hairpin? and detect? and target?
            9168  HAIRPIN?
         1563983  DETECT?
          422946  TARGET?
      S2     103  HAIRPIN? AND DETECT? AND TARGET?
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Processing
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 4/3,AB/1     (Item 1 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

09721546   98138682   PMID: 9478195
   Specific  cleavage of a target RNA from HIV-1 by mammalian tRNA 3'
processing endoribonuclease directed by an RNA heptamer.
  Nashimoto M; Kaspar R
  JT Life Science Research Laboratory, Kanagawa, Japan.
  Nucleic acids symposium series (ENGLAND)   1997,    (36)  p22-5,
ISSN 0261-3166   Journal Code: 8007206
  Document type: Journal Article
  Languages: ENGLISH
  Main Citation Owner: NLM
  Record type: Completed
  Mammalian  tRNA  3' processing endoribonuclease (3' tRNase) can recognize
pre-tRNA-like complexes between a 5' half tRNA(Arg) and a 3' half tRNA(Arg)
with  a  3'  trailer,  and  can  cleave  the  3'  half  tRNA(Arg) after the
discriminator  nucleotide  (Nashimoto M., 1996, RNA, 2:523-534). 3' tRNase
cleaved  the  3'  half  tRNA(Arg)  in  the  presence of a 7-nt 5' tRNA(Arg)
composed  only  of  the  acceptor  stem  region with a catalytic efficiency
comparable  to  that  of  cleavage directed by the intact 5' half tRNA(Arg).
The  catalytic efficiency of cleavage directed by the heptamer decreased as
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the stability of the T stem-loop structures of 3' half tRNA(Arg) variants decreased. No heptamer-directed cleavage of a 3' half tRNA(Arg) was **detected** in the absence of T-stem base pairs. This strategy for **targeted** RNA cleavage using an RNA heptamer was applied to the cleavage of two **target** RNAs from HIV-1. An HIV-1 RNA containing a stable **hairpin** structure corresponding to the T stem-loop was cleaved efficiently, whereas no cleavage of a second HIV-1 **target** without any stable **hairpin** structure was **detected**. In this method, an RNA heptamer can direct efficient RNA cleavage with a higher specificity than expected.

4/3,AB/2 (Item 2 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

09354431 97256756 PMID: 9099685
Characterization of the calf thymus **hairpin**-binding factor involved in histone pre-mRNA 3' end processing.
Schaller A; Martin F; Muller B
Abteilung Entwicklungsbiologie, Zoologisches Institut, Universitat Bern, Baltzerstrasse 4, CH 3012 Bern, Switzerland.
Journal of biological chemistry (UNITED STATES) Apr 18 **1997**, 272 (16) p10435-41, ISSN 0021-9258 Journal Code: 2985121R
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Using ion exchange chromatography we have enriched the RNA **hairpin** -binding factor involved in histone pre-mRNA processing from calf thymus whole cell extract. We demonstrate that the interaction of the factor with its **target** RNA sequence, the **hairpin** structure located at the 3' end of mature histone mRNA, is sequence-specific and highly salt-resistant. We have developed a simple in vitro system which allows **detection** of activities stimulating histone pre-mRNA 3' end processing, based on mouse cell nuclear extract fractionated by Mono Q column chromatography. Using this system, we show that the bovine **hairpin** -binding factor participates in histone pre-mRNA 3' end processing in vitro. We have further purified the **hairpin**-binding factor in form of a RNA.protein complex by RNA-mediated elution from phosphocellulose. This led to a fraction highly enriched for 2 proteins of 40 and 43 kDa.

4/3,AB/3 (Item 3 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

09268918 97151079 PMID: 8995615
Specific initiation of replication at the right-end telomere of the closed species of minute virus of mice replicative-form DNA.
Baldauf A Q; Willwand K; Mumtsidu E; Nuesch J P; Rommelaere J
Department of Applied Tumor Virology, Deutches Krebsforschungszentrum, Heidelberg, Germany.
Journal of virology (UNITED STATES) Feb **1997**, 71 (2) p971-80, ISSN 0022-538X Journal Code: 0113724
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
We have developed an in vitro system that supports the replication of natural DNA templates of the autonomous parvovirus minute virus of mice (MVM). MVM virion DNA, a single-stranded molecule bracketed by short, terminal, self-complementary sequences, is converted into double-stranded replicative-form (RF) DNA when incubated in mouse A9 fibroblast extract. The 3' end of the newly synthesized complementary strand is ligated to the

right-end **hairpin** of the virion strand, resulting in the formation of a covalently closed RF (cRF) molecule as the major conversion product. cRF DNA is not further replicated in A9 cell extract alone. On addition of purified MVM nonstructural protein NS1 expressed from recombinant baculoviruses or vaccinia viruses, cRF DNA is processed into a right-end (5' end of the virion strand) extended form (5'eRF). This is indicative of NS1-dependent nicking of the right-end **hairpin** at a distinct position, followed by unfolding of the **hairpin** and copying of the terminal sequence. In contrast, no resolution of the left-end **hairpin** can be **detected** in the presence of NS1. In the course of the right-end nicking reaction, NS1 gets covalently attached to the right-end telomere of the DNA product, as shown by immunoprecipitation with NS1-specific antibodies. The 5'eRF product is the **target** for additional rounds of NS1-induced nicking and displacement synthesis at the right end, arguing against the requirement of the **hairpin** structure for recognition of the DNA substrate by NS1. Further processing of the 5'eRF template in vitro leads to the formation of dimeric RF (dRF) DNA in a left-to-left-end configuration, presumably as a result of copying of the whole molecule by displacement synthesis initiated at the right-end telomere. Formation of dRF DNA is highly stimulated by NS1. The experimental results presented in this report support various assumptions of current models of parvovirus DNA replication and provide new insights into the replication functions of the NS1 protein.

4/3,AB/4 (Item 4 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

09268828 97151099 PMID: 8995635
A novel terminal resolution-like site in the adeno-associated virus type 2 genome.
Wang X S; Srivastava A
Department of Medicine, Walther Oncology Center, Indiana University School of Medicine, Indianapolis 46202, USA.
Journal of virology (UNITED STATES) Feb **1997**, 71 (2) p1140-6, ISSN 0022-538X Journal Code: 0113724
Contract/Grant No.: DK-49218; DK; NIDDK; HL-48342; HL; NHLBI; HL-53586; HL; NHLBI
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
The adeno-associated virus 2 (AAV) contains a single-stranded DNA genome of which the terminal 145 nucleotides are palindromic and form T-shaped **hairpin** structures. These inverted terminal repeats (ITRs) play an important role in AAV DNA replication and resolution, since each of the ITRs contains a terminal resolution site (trs) that is the **target** site for the AAV rep gene products (Rep). However, the Rep proteins also interact with the AAV DNA sequences that lie outside the ITRs, and the ITRs also play a crucial role in excision of the proviral genome from latently infected cells or from recombinant AAV plasmids. To distinguish between Rep-mediated excision of the viral genome during rescue from recombinant AAV plasmids and the Rep-mediated resolution of the ITRs during AAV DNA replication, we constructed recombinant AAV genomes that lacked either the left or the right ITR sequence and one of the Rep-binding sites (RBSs). No rescue and replication of the AAV genome occurred from these plasmids following transfection into adenovirus type 2-infected human KB cells, as expected. However, excision and abundant replication of the vector sequences was clearly **det cted** from the plasmid that lacked the AAV left ITR, suggesting the existence of an additional putative excision site in the left end of the AAV genome. This site was precisely mapped to one of the AAV promoters at map unit 5 (AAV p5) that also contains an RBS. Furthermore, deletion of this RBS abolished the rescue and replication of the vector sequences. These studies suggest that the Rep-mediated cleavage

at the RBS during viral DNA replication may, in part, account for the generation of the AAV defective interfering particles.

4/3,AB/5 (Item 5 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

08825584 96183286 PMID: 8605174
In vitro selection analysis of neomycin binding RNAs with a mutagenized pool of variants of the 16S rRNA decoding region.
Famulok M; Huttenhofer A
Institut fur Biochemie der Ludwig-Maximilians-Universitat Munchen, Germany.
Biochemistry (UNITED STATES) Apr 9 **1996**, 35 (14) p4265-70, ISSN 0006-2960 Journal Code: 0370623
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
An in vitro selection for neomycin B binding was carried out with an RNA pool containing a 47-nucleotide domain of the decoding region of 16S ribosomal RNA, mutated at 30% per base position. The degenerate region was comprised of an oligonucleotide analogue ("motif A") of the decoding region in 30S subunits which has previously been shown to interact with the aminoglycoside antibiotic neomycin B and tRNA ligands. After five cycles of selection/amplification, RNA sequences were isolated which specifically bound to neomycin B. Cloning and sequencing showed that none of the isolated clones shared primary sequence or secondary structure homology with the decoding region of 16S RNA. Instead, a new set of sequences was isolated which could be folded into a defined **hairpin** structure designated as motif B. We investigated the affinity of motif A, motif B, the unselected pool RNA, and the corresponding unmutagenized "parent" RNA to neomycin B at different Mg2+ concentrations. Under buffer conditions of low ionic strength all RNAs tested bound nonspecifically to neomycin B. However, motif B bound to neomycin B at Mg2+ concentrations at which binding of the other RNAs tested was significantly lower or not **detectable** . This is consistent with motif B exhibiting a higher affinity for neomycin B than motif A under these conditions. Motif B has previously been isolated from an in vitro selection to identify RNA sequences with affinity to neomycin B using a completely randomized RNA pool which shared no relationship to motif A. Our results indicate that motif B might represent a highly optimized RNA sequence for neomycin B binding; conversely, the A-site motif in 16S rRNA (motif A) might not be an optimal **target** for neomycin B recognition.

4/3,AB/6 (Item 6 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

08681929 96050918 PMID: 7584058
Intracellular immunization of human T cells with a **hairpin** ribozyme against human immunodeficiency virus type 1.
Yamada O; Yu M; Yee J K; Kraus G; Looney D; Wong-Staal F
Department of Medicine, University of California, San Diego, La Jolla 92093-0665, USA.
Gene therapy (ENGLAND) Jan **1994**, 1 (1) p38-45, ISSN 0969-7128 Journal Code: 9421525
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
T-cell lines (Jurkat and Molt-4) were transduced with retroviral vectors containing a **hairpin** ribozyme that **targets** a conserved sequence in the 5' transcribed leader sequence of human immunodeficiency virus (HIV)

type 1. Stable cell lines were generated which constitutively and persistently expressed the ribozyme gene driven by either the Moloney retroviral long terminal repeat (LTR) or an internal human tRNA(val) promoter. There was no apparent deleterious effect of long-term ribozyme expression on cell proliferation or viability. Cells expressing ribozyme were resistant to challenge from diverse strains of HIV, including an uncloned clinical isolate. No reverse transcriptase activity or virus infectivity was **detectable** in the culture supernatants of Jurkat cells expressing the ribozyme driven by the tRNA(val) promoter up to 35 days after challenge with HIV-1/HXB2. Expression of the ribozyme also significantly decreased (by approximately 50- to 100-fold) the efficiency of incoming virus to synthesize viral DNA. These and previously reported results indicate that transfer and expression of the ribozyme gene interfere with both early and late events in the HIV replication cycle and confer long-term resistance to HIV-1 infection.

4/3,AB/7 (Item 7 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

08636298 95395861 PMID: 7666435
DNA palindromes adopt a methylation-resistant conformation that is consistent with DNA cruciform or **hairpin** formation in vivo.
Allers T; Leach D R
Institute of Cell and Molecular Biology, University of Edinburgh, UK.
Journal of molecular biology (ENGLAND) Sep 8 **1995**, 252 (1) p70-85, ISSN 0022-2836 Journal Code: 2985088R
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Long DNA palindromes present a threat to genomic stability and are not tolerated in Escherichia coli. It has been suggested that this is a consequence of cruciform or **hairpin** formation by palindromic sequences. This work describes a methylation inhibition assay for unusual DNA secondary structure in vivo that is both internally controlled and non-invasive. If a palindrome with a central GATC **target** site for Dam methylase assumes a cruciform or **hairpin** conformation in vivo, then the GATC sequence will be located in a single-stranded loop and will consequently not be modified. The centre of a long perfect palindrome located in bacteriophage lambda is shown to be methylation-resistant in vivo. Changes to the central sequence and insertions of 10 base-pairs of asymmetric sequence do not alter the degree of under-methylation, but insertions of 20 base-pairs or more of asymmetric sequence reduce the under-methylation of the palindrome centre. We also show that the centres of long palindromes are more under-methylated than equivalent sequences in a non-palindromic context. These results are consistent with an unusual secondary structure, such as DNA cruciform or **hairpin**, and indicate that the formation pathway of the structure **detected** is independent of the composition and symmetry of the central 10 base-pairs of the palindrome.

4/3,AB/8 (Item 8 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

08449691 95198724 PMID: 7891700
Fine mapping of 28S rRNA sites specifically cleaved in cells undergoing apoptosis.
Houge G; Robaye B; Eikhom T S; Golstein J; Mellgren G; Gjertsen B T; Lanotte M; Doskeland S O
Department of Anatomy and Cell Biology, University of Bergen, Norway.
Molecular and cellular biology (UNITED STATES) Apr **1995**, 15 (4) p2051-62, ISSN 0270-7306 Journal Code: 8109087

Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed

Bona fide apoptosis in rat and human leukemia cells, rat thymocytes, and bovine endothelial cells was accompanied by limited and specific cleavage of polysome-associated and monosome-associated 28S rRNA, with 18S rRNA being spared. Specific 28S rRNA cleavage was observed in all instances of apoptotic death accompanied by internucleosomal DNA fragmentation, with cleavage of 28S rRNA and of DNA being linked temporally. This indicates that 28S rRNA fragmentation may be as general a feature of apoptosis as internucleosomal DNA fragmentation and that concerted specific cleavage of intra- and extranuclear polynucleotides occurs in apoptosis. Apoptosis-associated cleavage sites were mapped to the 28S rRNA divergent domains D2, D6 (endothelial cells), and D8. The D2 cuts occurred in **hairpin** loop junctions considered to be buried in the intact ribosome, suggesting that this rRNA region becomes a **target** for RNase attack in apoptotic cells. D8 was cleaved in two exposed UU(U) sequences in bulge loops. Treatment with agents causing necrotic cell death or aging of cell lysates failed to produce any **detectable** limited D2 cleavage but did produce a more generalized cleavage in the D8 region. Of potential functional interest was the finding that the primary cuts in D2 exactly flanked a 0.3-kb hypervariable subdomain (D2c), allowing excision of the latter. The implication of hypervariable rRNA domains in apoptosis represents the first association of any functional process with these enigmatic parts of the ribosomes.

4/3,AB/9 (Item 9 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

08306013 95065643 PMID: 7975207
Activity and cleavage site specificity of an anti-HIV-1 **hairpin** ribozyme in human T cells.
Yamada O; Kraus G; Leavitt M C; Yu M; Wong-Staal F
Department of Medicine and Biology, University of California, San Diego 92093-0665.
Virology (UNITED STATES) Nov 15 **1994**, 205 (1) p121-6, ISSN 0042-6822 Journal Code: 0110674
Contract/Grant No.: AI 31378; AI; NIAID
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed

Human CD4+ T cells (Molt-4) were transduced with retroviral vectors containing a **hairpin** ribozyme which **targets** the rev/env coding region of HIV-1 RNA (HXB2: 8629-8644). This **target** sequence is conserved among many HIV-1 clones, including the prototype virus HXB2, but the infectious clone SF2 contains a single nucleotide substitution at the cleavage site (from N*GUC to N*UUC). Cells stably expressing the ribozyme or its disabled counterpart were challenged with HXB2 or SF2 and the amount of p24 antigen produced was monitored. While this ribozyme was effective in inhibiting the replication of HXB2 in Molt 4 cells, it showed only marginal inhibitory effect on SF2 replication. The same level of virus production was observed with cells transduced by the disabled ribozyme, which functions essentially as an antisense molecule. Expression of the ribozyme was comparable in HXB2- or SF2-infected cells as **detected** by reverse transcription-polymerase chain reaction. These data provide in vivo evidence that the antiviral activity of the **hairpin** ribozyme is strictly dependent on the presence of the cleavage site in the **target** RNA and supports the conclusion that the ribozyme acts as catalytic RNA rather than as antisense RNA in vivo.

4/3,AB/10 (Item 10 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

07854620 93386894 PMID: 8375077
A transcriptionally amplified DNA probe assay with ligatable probes and immunochemical **detection**.
Carpenter W R; Schutzbank T E; Tevere V J; Tocyloski K R; Dattagupta N; Yeung K K
Miles Inc., Diagnostics Division, Tarrytown, NY 10591.
Clinical chemistry (UNITED STATES) Sep **1993**, 39 (9) p1934-8, ISSN 0009-9147 Journal Code: 9421549
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Transcriptionally amplified DNA probes are valuable tools in the development of sensitive nucleic acid-based diagnostic assays. Here we describe a model assay using a novel oligonucleotide **hairpin** probe that encodes a T7 RNA polymerase promoter. The **hairpin** probe and an adjacently hybridizing biotinylated capture probe were hybridized to **target** DNA and the duplex was captured onto streptavidin-coated magnetic particles. After ligation of the immobilized probes, which served to maintain specificity, the **hairpin** probe was transcribed by T7 RNA polymerase. The amplified RNA product was hybridized to the capture probe and bound to the streptavidin-coated magnetic particles. The immobilized heteroduplex was **detected** with an antibody-alkaline phosphatase conjugate specific for DNA:RNA hybrids, and the chemiluminescent substrate adamantyl-1,2-dioxetane phenyl phosphate. Ten attomoles of **target** DNA could be **detected** in a background of 5 micrograms of unrelated DNA. The chemiluminescent immunoassay was as sensitive as radioactive **detection** of specific product after gel electrophoresis.

4/3,AB/11 (Item 11 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

07827196 93360262 PMID: 7689111
Erythromycin binding is reduced in ribosomes with conformational alterations in the 23 S rRNA peptidyl transferase loop.
Douthwaite S; Aagaard C
Department of Molecular Biology, Odense University, Denmark.
Journal of molecular biology (ENGLAND) Aug 5 **1993**, 232 (3) p725-31, ISSN 0022-2836 Journal Code: 2985088R
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
The antibiotic erythromycin inhibits protein synthesis by binding to the 50 S ribosomal subunit, where the drug interacts with the unpaired bases 2058A and 2059A in the peptidyl transferase loop of 23 S rRNA. We used a chemical modification approach to analyse conformational changes that are induced by mutations in the peptidyl transferase loop, and to determine how these changes affect drug interaction. Mutations at positions 2057 (G-->A) and 2058 (A-->G, or -->U), all of which confer drug resistance, induce a more open conformation in the peptidyl transferase loop. Erythromycin still protects against chemical modification in the mutant peptidyl transferase loops, but the affinity of the drug interaction is reduced 20-fold in the 2057A mutant, 10(3)-fold in the 2058U mutant and 10(4)-fold in the 2058G mutant. Single mutations at position 2032 in the adjacent **hairpin** loop, which have previously been shown to alter drug tolerances, gave no **detectable** effects on the structure of the peptidyl transferase loop or on erythromycin binding. Dual mutations at positions 2032 and 2058, however, induce a marked change in the rRNA conformation with opening of the phylogenetically conserved base-pair 2063C.2447G, and confer a slow

growth, drug-sensitive phenotype. The data suggest that the **target** site of erythromycin lies within the peptidyl transferase loop, and that limited disruption of the conformation of this site reduces drug binding, and consequently confers resistance. In addition, there is structurally and functionally important interaction between the drug **target** site in the peptidyl transferase loop and position 2032.

4/3,AB/12 (Item 12 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

07727774 93252944 PMID: 8486707
EBP-80, a transcription factor closely resembling the human autoantigen Ku, recognizes single- to double-strand transitions in DNA.
Falzon M; Fewell J W; Kuff E L
Laboratory of Biochemistry, National Cancer Institute, Bethesda, Maryland 20892.
Journal of biological chemistry (UNITED STATES) May 15 **1993**, 268 (14) p10546-52, ISSN 0021-9258 Journal Code: 2985121R
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
We have previously reported the purification and characterization of the transcription factor EBP-80 (Falzon, M., and Kuff, E. L. (1989) J. Biol. Chem. 264, 21915-21922). EBP-80 mediates the DNA methylation effect on transcription from an endogenous proviral long terminal repeat. Here we show that EBP-80 is very similar if not identical to the Ku autoantigen, a heterodimeric nuclear protein first **detected** by antibodies from autoimmune patients (Mimori, T., Akizuki, M., Yamagata, H., Inada, S., Yoshida, S., and Homma, M. (1981) J. Clin. Invest. 68, 611-620). A number of laboratories have shown that the Ku protein complex binds to free double-stranded DNA ends. In this study, we have examined the binding properties of EBP-80. EBP-80 binds single-stranded DNA with low affinity. Binding to random sequence double-stranded DNA depends on the length of the duplex and is optimal with oligomers of 30 and 32 base pairs; the protein complexes formed with these oligomers have Kd values of 15-20 pM. It binds with comparable high affinities to blunt-ended duplex DNA, to duplex DNA ending in **hairpin** loops, and to constructs in which an internal segment of duplex DNA is flanked by single-strand extensions. EBP-80 also interacts effectively with circular duplex molecules containing a 30-nucleotide single-stranded region (gap) or a double-stranded segment of nonhomology (bubble), but only weakly with the corresponding closed circular construct made up entirely of duplex DNA. EBP-80 prefers A/T to G/C ends. The binding properties of EBP-80 are consistent with the hypothesis that is recognizes single- to double-strand transitions in DNA. A model is presented for the interaction of EBP-80 with its **target** sequence in the proviral long terminal repeat.

4/3,AB/13 (Item 13 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

07088353 92020138 PMID: 1656384
RNA--protein interactions within the internal translation initiation region of encephalomyocarditis virus RNA.
Borovjagin A V; Ezrokhi M V; Rostapshov V M; Ugarova TYu; Bystrova T F; Shatsky I N
A.N. Belozersky Laboratory, Moscow State University, USSR.
Nucleic acids research (ENGLAND) Sep 25 **1991**, 19 (18) p4999-5005, ISSN 0305-1048 Journal Code: 0411011
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM

Record type: Completed
Various derivatives of the internal ribosomal entry site (IRES) of encephalomyocarditis virus (EMCV) RNA have been used to analyze by UV-cross-linking its interaction with mRNA binding proteins from ascites carcinoma Krebs-2 cells. A doublet of proteins with Mr 58 and 60 kD bound to two regions of the IRES. One site is centered at nt 420-421 of EMCV RNA whereas the other is located between nt 315-377. Both sites form **hairpin** structures, the loops of which contain UCUUU motif, conserved among cardio- and aphthoviruses. The interaction of p58 and p60 with IRES is affected by the integrity of the stem-loop structure proximal to the start AUG codon (nts 680-787), although, under similar conditions, cross-linking of these proteins to this region was not **detected**. Deletions in the main recognition site of p58 strongly reduce the initiation activity of the IRES in vitro. However, elimination of p58 (p60) binding by these mutations does not completely abolish the ability of the IRES to direct polypeptide synthesis starting from the authentic AUG codon. The IRES can be assembled in vitro from two covalently unlinked transcripts, one containing the **target** site for p58 and the other encompassing the remaining part of the IRES fused to a reporter gene, resulting in considerable restoration of its activity. Implications of these findings for the mechanism of initiation resulting from internal entry of ribosomes are discussed.

4/3,AB/14 (Item 14 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

06222915 89307123 PMID: 2744707
Hairpin extension. A general method for the improvement of sensitivity of oligonucleotide probes.
Sriprakash K S; Hartas J
Menzies School of Health Research, Darwin, N.T., Australia.
Gene analysis techniques (UNITED STATES) Mar-Apr **1989**, 6 (2) p29-32, ISSN 0735-0651 Journal Code: 8408118
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
A general and sensitive **detection** method of **target** DNA is described. The system is based on an oligonucleotide probe labeled to high specific activity. This involves a novel oligonucleotide design incorporating at the 3' end a **hairpin** structure, allowing extension by polymerase reaction.

4/3,AB/15 (Item 15 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

05956441 89042141 PMID: 3054882
Frequency and spectrum of mutations produced by a single cis-syn thymine-thymine cyclobutane dimer in a single-stranded vector.
Banerjee S K; Christensen R B; Lawrence C W; LeClerc J E
Department of Biophysics, University of Rochester School of Medicine and Dentistry, NY 14642.
Proceedings of the National Academy of Sciences of the United States of America (UNITED STATES) Nov **1988**, 85 (21) p8141-5, ISSN 0027-8424 Journal Code: 7505876
Contract/Grant No.: GM21858; GM; NIGMS; GM32885; GM; NIGMS
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
We have constructed a single-stranded vector that contains a uniquely located cis-syn T-T cyclobutane dimer by ligating a synthetic oligomer

containing this UV photoproduct into M13mp7 viral DNA linearized with EcoRI. In the absence of SOS induction, transfection of a uvrA6 mutant of Escherichia coli with this vector gave very few progeny plaques, and the data imply that a single dimer blocks replication in at least 99.5% of the molecules. In vitro photoreactivation completely abolished this inhibition. Transfection of cells irradiated with UV at 4 J.m-2 to induce the SOS response gave 27% of the number of plaques found with a dimer-free control. Nucleotide sequence analysis of 529 progeny phage showed that translesion synthesis was usually accurate: the normal sequence was found in 93% of them. Where mutations occurred, all were **targeted** single-nucleotide substitutions, with approximately 90% being **targeted** at the 3' nucleotide of the lesion: of a total of 26 mutations, 15 were 3' T----A, 8 were 3' T----C, and 3 were 5' T----C. No T----G mutations were found. In addition to these results with the normal construct, data were also obtained from vectors in which the M13mp7 cloning site, which forms a **hairpin** in single-stranded DNA, was present 4 nucleotides on the 3' side of the T-T dimer. These **hairpin**-containing vectors gave a very similar mutation frequency (8% versus 7%) but altered mutation spectrum: all 12 mutations **detected** were 3' T----A transversions, a difference from the previous set of data that is significant (P = 0.03).

4/3,AB/16 (Item 16 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

05447017 87198947 PMID: 3033323

Efficient resolution of replicated poxvirus telomeres to native **hairpin** structures requires two inverted symmetrical copies of a core **target** DNA sequence.

DeLange A M; McFadden G

Journal of virology (UNITED STATES) Jun **1987**, 61 (6) p1957-63, ISSN 0022-538X Journal Code: 0113724
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed

The terminal **hairpin** sequences of the linear double-stranded DNA genome of the leporipoxvirus Shope fibroma virus (SFV) has been cloned in Saccharomyces cerevisiae and in recombination-deficient Escherichia coli as a palindromic insert within circular plasmid vectors. This sequence configuration is equivalent to the inverted repeat structure **detected** as a telomeric replicative intermediate during poxvirus replication in vivo. Previously, it has been shown that when circular plasmids containing this palindromic insert were transfected into SFV-infected cells, efficient replication and resolution generated linear minichromosomes with bona fide viral **hairpin** termini (A. M. DeLange, M. Reddy, D. Scraba, C. Upton, and G. McFadden, J. Virol. 59:249-259, 1986). To localize the minimal **target** DNA sequence required for efficient resolution, a series of staggered unidirectional deletions were constructed at both ends of the inverted repeat. Analyses of the resolution efficiencies of the various clones indicate that up to 240 base pairs (bp) centered at the symmetry axis were required for maximal resolution to minichromosomes. To investigate the role of the AT-rich central axis sequences, which in SFV include 8 nonpalindromic bp, a unique AflII site at the symmetry axis was exploited. Bidirectional deletions extending from this AflII site and insertions of synthetic oligonucleotides into one of the deletion derivatives were constructed and tested in vivo. The efficiency with which these plasmids resolved to linear minichromosomes with **hairpin** termini has enabled us to define the minimal **target** DNA sequence as two inverted copies of an identical DNA sequence between 58 and 76 bp in length. The nonpalindromic nucleotides, which, after resolution, constitute the extrahelical residues characteristic of native poxviral telomeres, were not required for resolution. The close resemblance of the SFV core **target** sequence to the analogous region from the orthopoxvirus

vaccinia virus is consistent with a conserved mechanism for poxviral telomere resolution.

4/3,AB/17 (Item 17 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

04688408 85061111 PMID: 6209541
Sequence specificities in the interactions of chemicals and radiations with DNA.
Wilkins R J
Molecular and cellular biochemistry (NETHERLANDS) Sep **1984**, 64 (2) p111-26, ISSN 0300-8177 Journal Code: 0364456
Document type: Journal Article; Review
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Sequence specificities in the interactions of chemicals and radiations with DNA are reviewed. Emphasis is placed on information which has been obtained by adapting DNA sequencing techniques to the **detection** of DNA damage and modifications. The actions of anti-tumor drugs, non-covalent DNA binders and UV irradiation are discussed in terms of both modifications induced in DNA and the subsequent response of DNA polymerase and repair enzymes. Particular attention is paid to the evidence for 'sequence-specific' interactions of these agents with DNA. It is concluded that while most agents exhibit 'warm' or even 'hot' spots in their interactions with DNA there is not, as yet, compelling evidence for extreme selectivity down to say the gene level in their actions. There does, however, appear to be some affinity, particularly in the case of non-covalent binders, for certain tertiary structures rather than primary sequences per se. In addition, both misincorporation opposite DNA damage and the bypass of damage by polymerases are important phenomena which, to some extent, exhibit sequence specificities. The idea is advanced that DNA structures such as **hairpins** or cruciforms maybe important in vivo **targets** for many agents giving rise to specific biological effects.

4/3,AB/18 (Item 1 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

09439906 BIOSIS NO.: 199497448276
Strand displacement amplification as an in vitro model for rolling-circle replication: Deletion formation and evolution during serial transfer.
AUTHOR: Walter Nils G(a); Strunk Gunther
AUTHOR ADDRESS: (a)Dep. Biochem. Kinetics, Max Planck Inst. Biophysical Chem., Am Fasberg 11, D-37077 Goettingen**Germany
JOURNAL: Proceedings of the National Academy of Sciences of the United States of America 91 (17):p7937-7941 **1994**
ISSN: 0027-8424
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: Strand displacement amplification is an isothermal DNA amplification reaction based on a restriction endonuclease nicking its recognition site and a polymerase extending the nick at its 3' end, displacing the downstream strand. The reaction resembles rolling-circle replication of single-stranded phages and small plasmids. The displaced sense strand serves as **target** for an antisense reaction and vice versa, resulting in exponential growth and the autocatalytic nature of this in vitro reaction as long as the template is the limiting agent. We describe the optimization of strand displacement amplification for in vitro evolution experiments under serial transfer conditions. The

reaction was followed and controlled by use of the fluorescent dye thiazole orange binding to the amplified DNA. We were able to maintain exponential growth conditions with a doubling time of 3.0 min throughout 100 transfers or apprxeq 350 molecular generations by using an automatic handling device. Homology of in vitro amplification with rolling-circle replication was mirrored by the occurring evolutionary processes. Deletion events most likely caused by a slipped mispairing mechanism as postulated for in vivo replication took place. Under our conditions, the mutation rate was high and a molecular quasi-species formed with a mutant lacking internal **hairpin** formation ability and thus outgrowing all other species under dGTP/dCTP deficiency.

1994

4/3,AB/19 (Item 2 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

08969263 BIOSIS NO.: 199396120764
Erythromycin binding is reduced in ribosomes with conformational alterations in the 23S rRNA peptidyltransferase loop.
AUTHOR: Douthwaite Stephen; Aagaard Claus
AUTHOR ADDRESS: Dep. Molecular Biol., Odense Univ., DK-5230 Odense M** Denmark
JOURNAL: Journal of Molecular Biology 232 (3):p725-731 **1993**
ISSN: 0022-2836
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: The antibiotic erythromycin inhibits protein synthesis by binding to the 50 S ribosomal subunit, where the drug interacts with the unpaired bases 2058A and 2059A in the peptidyl transferase loop of 23 S rRNA. We used a chemical modification approach to analyse conformational changes that are induced by mutations in the peptidyl transferase loop, and to determine how these changes affect drug interaction. Mutations at positions 2057 (G fwdarw A) and 2058 (A fwdarw G, or fwdarw U), all of which confer drug resistance, induce a more open conformation in the peptidyl transferase loop. Erythromycin still protects against chemical modification in the mutant peptidyl transferase loops, but the affinity of the drug interaction is reduced 20-fold in the 2057A mutant, 10-3-fold in the 2058U mutant and 10-4-fold in the 2058G mutant. Single mutations at position 2032 in the adjacent **hairpin** loop, which have previously been shown to alter drug tolerances, gave no **detectable** effects on the structure of the peptidyl transferase loop or on erythromycin binding. Dual mutations at positions 2032 and 2058, however, induce a marked change in the rRNA conformation with opening of the phylogenetically conserved base-pair 2063C cntdot 2447G, and confer a slow growth, drug-sensitive phenotype. The data suggest that the **target** site of erythromycin lies within the peptidyl transferase loop, and that limited disruption of the conformation of this site reduces drug binding, and consequently confers resistance. In addition, there is structurally and functionally important interaction between the drug **target** site in the peptidyl transferase loop and position 2032.

1993